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Host-parasite interactions of *Neospora caninum*

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Dedication

I dedicate this thesis to my family and to those that we have lost.

"*Gone but never forgotten"*

Declaration

I declare that I composed and wrote this thesis and that it describes my research.

Paul Murdoch Bartley

31st March 2016

Acknowledgments

I would like to start by thanking Prof Elisabeth A Innes and Dr. Frank Katzer for their friendship, guidance and mentoring over so many years. Without the faith that they have shown in me the projects included in this thesis would not have been possible. Thanks to both for all of their help and comments on the manuscripts included here and for making me look at my work in a more critical way.

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Abstract

The papers included in this thesis examine the host–parasite relationship in small and large animals following experimental challenges with *Neospora caninum*. This apicomplexan parasite is a major cause of abortion and reproductive losses in cattle worldwide. Economic and welfare issues make the development of a vaccine against the transplacental transmission of *Neospora* highly desirable.

This thesis evaluates the host-parasite interactions in a non-pregnant mouse model examining whether the actively multiplying stage of the parasite (tachyzoite) could be attenuated through prolonged *in vitro* cultivation (passage) and used as a live vaccine. We show that continued maintenance of tachyzoites in tissue culture produced significantly reduced levels of morbidity and mortality in the mice following challenge, compared to mice receiving virulent parasites. Inoculation with a sub-lethal dose of tachyzoites was shown to protect against a subsequent lethal challenge of virulent parasites. Mice showing higher levels of cell mediated immunity (CMI) (antigen-specific splenocyte proliferation and interferon-γ (IFN-γ) production) had lower parasite burdens compared to mice with less pronounced CMI responses. Combined, these works show that it is possible to protect against a lethal challenge using attenuated tachyzoites and that a strong T-helper Type-1 CMI response is involved in protection and in reducing clinical disease severity.

As the most commonly known route of infection with *N. caninum* is transplacental, from dam to foetus, we also wanted to examine the host-parasite relationship in pregnant

cattle. This was done through the serial examination of the maternal and foetal immune responses of experimentally challenged cattle under controlled conditions at different stages throughout pregnancy. These works show the importance of the timing, location and magnitude of multiple components of the host immune response in determining foetal survival and also whether vertical transmission occurs. We show that both the maternal and foetal immune responses are critical in determining the clinical outcome of infection. A strong maternal CMI response was shown to aid foetal survival by reducing the numbers of parasites reaching and thus damaging the placenta. Due to the syndesmychorial nature of the ruminant placenta, any foetal responses observed are as a result of foetal infection. These experiments show that as pregnancy progresses the foetus goes from being immunologically immature and incapable of mounting a protective immune response (70 days of gestation (dg)) to becoming capable of mounting parasite-specific humoral, innate and CMI responses from around 140dg onwards.

The experiments in pregnant cattle confirm the importance of parasite-specific proliferation and IFN-γ production, in reducing the magnitude of the parasite challenge reaching the maternal–foetal interface and aiding foetal survival. We also examined the immunodominant parasite peptides expressed in HPLC fractionated tachyzoite antigen, which are recognised by the cellular immune response of experimentally challenged cattle. Through LC-ESI-MS/MS, 6 *Neospora* proteins (including SAG1, SRS2 and GRA2) and a number of *Toxoplasma gondii* orthologues were identified and found to be recognised by CD4+ T-cells.

These works collectively demonstrate the complexity of the host-parasite interaction in *Neospora* infections and show the importance of a CMI response in protection against the parasite.

Lay summary

Neospora caninum is a parasite that causes abortions in cows, sheep and goats all around the world. The work in this thesis starts by looking at what happens when mice get infected with the parasite. Some parasites can actually help protect animals against the more harmful parasites. These protected mice have more white blood cells helping to fight off the parasites, meaning that they get less sick and get better quicker.

I have also looked at what happens when pregnant cows get infected with the parasite. As the parasite can cross the placenta from mum to baby, we wanted to look at what happens to the baby if the parasite gets across the placenta. We looked at the white blood cells that helped fight off the parasite at different points, at early, middle and late pregnancy. In early pregnancy when baby is still very small it cannot fight off the parasite and sadly can die. However as the baby gets bigger at middle and late pregnancy, it is able to fight off the parasite and is more likely to survive.

When looking at the types of white blood cells that help fight off the parasite, we wanted to find out which parasite proteins (antigens) the white blood cells recognised and which ones may help produce an immune response and could possibly be used to make a vaccine against the parasite.

The results from this thesis have helped us to understand how the immune systems of mice and cows respond if they are infected with *Neospora*. This information will help in developing a possible vaccine against the parasite.

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1 General introduction

Neospora caninum is considered to be one of the most commonly diagnosed causes of infectious bovine abortion and foetal death worldwide. Neosporosis is estimated to cause US$ 1.3 billion in losses annually worldwide, across the beef and dairy industries (Reichel et al., 2012). There are currently no commercially available vaccines, or licensed chemotherapeutics that can clear *Neospora* infections in cattle. As a result, farmers are reliant solely upon accurate diagnosis coupled with good animal husbandry and biological control strategies, to limit the spread of the parasite. Improved biological control strategies and the development of an effective vaccine are highly desirable aims, which will ultimately limit the economic impact of *Neospora* and improve farming efficiency. However to progress vaccine development and other control strategies, we require a better understanding of the aetiology of Neosporosis and the host-parasite interactions. When embarking on the body of work that comprises this thesis, the understanding in a number of critical areas was lacking and this thesis has sought to address several of these gaps.

The processes involved in the initiation and maintenance of protective immune responses were not well understood. Mouse and cattle models were employed to examine in detail the host responses following a primary infection with *Neospora caninum*. Areas of interest included the involvement of toll like receptors and pro-inflammatory cell-mediated (Th1 type) immune responses in protection against the

parasite. Identifying the protective responses against *Neospora* are important to ensure that any future vaccine stimulates an appropriate and proportionate response.

Previous work has demonstrated that the bovine foetus was capable of mounting a protective immune response at mid-gestation (Day 140 of gestation) (Bartley et al., 2004). In this thesis, I sought to further clarify at what stage of development the bovine foetus becomes capable of mounting a protective *in utero* immune response against *Neospora*. Furthermore it is also important to establish whether the timing of a primary *Neospora* infection is critical to foetal survival and parasite transmission, as this will have implications for control strategies.

It is likely that vaccination will be the most effective strategy for controlling *Neospora* transmission in cattle. Live attenuated vaccines have been shown to be successful in controlling other protozoan parasites, (e.g. Toxovax for the control of *Toxoplasma gondii* abortion in sheep (Buxton, 1993) and Paracox a live attenuated *Eimeria* vaccine used in chickens (Williams, 2002)*;* Using a mouse model, I evaluated tissue-culture attenuated *Neospora* tachyzoites for their ability to generate a protective immune response against a subsequent lethal challenge with virulent parasite. In addition, I have sought to identify the immune-reactive components of a soluble fraction of *Neospora* tachyzoites that may be involved in protection and possible vaccine candidates.

Together this body of work has significantly improved our understanding of the host-parasite interactions of *Neospora caninum.* Of particularly importance is the

identification of the protective components of the host immune response and the critical nature of the timing of a primary infection, with respect to foetal survival and parasite transmission. This knowledge combined with the proteomic identification of vaccine candidates and the *in vivo* testing of a live attenuated strain of *Neospora* have added to the evidence indicating that vaccination may be a viable option for *Neospora* control.

1.1 Discovery and identification of *Neospora caninum*

Neospora caninum is an obligate intracellular protozoan parasite from the phylum apicomplexa, family Sarcocystidae, which was first described by Bjerkas and colleagues in 1984, as an unidentified cyst-forming sporozoon causing encephalomyelitis and myositis in dogs (Bjerkas et al., 1984). Dubey and colleagues (1988) formally identified the parasite, through the examination of histological sections of dogs that had suffered a fatal *Toxoplasma*-like illness. These examinations revealed an organism that was structurally distinct from *Toxoplasma gondii* and all other coccidian parasites. As a result of these finding the parasite was allocated a new genus and species, becoming *Neospora caninum* (Dubey et al., 1988a).

Since its first identification and isolation in dogs, *N. caninum* has been shown to be a major cause of abortions and reproductive losses in cattle worldwide (Dubey, 2003). Cases of bovine neosporosis have been reported in Europe (Stenlund et al., 1997), North and South America (Macedo et al., 2013, Pare et al., 1995), Africa (Jardine and Last,

1993) and Australasia (Miller et al., 2002). Natural *N. caninum* infections have also been reported in many other intermediate host species including sheep (Dubey and Lindsay, 1990), goats (Barr et al., 1992), deer (Dubey et al., 1996) and horses (Dubey, 2003), though cases in these species are not as numerous or economically important as infections in cattle.

What remains poorly understood are the factors that dictate whether or not abortion or the vertical transmission of the parasite occurs in cattle following an infection with *N. caninum*? Generally, pathogen induced abortions occur when a foetus and / or placenta becomes so damaged that foetal viability can no longer be maintained. It is clear that some cattle abort or vertically transmit the parasite, when under similar conditions others do not? Possible explanations for the differences in infection outcome, including maternal and foetal immune responses, foetal gestational age, parasite dose and strain virulence are examined further throughout this thesis.

1.1.1 Initial isolation of *Neospora caninum* (NC1) parasites into tissue culture

The first *Neospora* parasites (isolate NC1), which are used in all of the experiments included in this thesis, were isolated from naturally infected Labrador Retriever pups by Dubey and colleagues (1988b). Tissue homogenates from the pups were inoculated *in vitro* onto bovine cardiopulmonary arterial endothelial (CPAE) cell monolayers, which lead to the formation of tachyzoites (rapidly multiplying, actively invasive forms of the parasite, which are discussed in more detail in section 1.3.1) being seen within 5 days post inoculation. *In vivo* inoculation of Swiss white mice (n = 25) and beagle pups (n =

2) with the same Labrador pup tissue (NC1) homogenates resulted in the formation of tissue cysts containing bradyzoites (see section 1.3.2) in the brains of a number of the mice. But no demonstrable tachyzoites were found in the beagle pups (Dubey et al., 1988b). A further beagle pup was inoculated with the *in vitro* culture derived NC1 tachyzoites. This NC1 tachyzoite challenge resulted in an acute infection in the pup; leading to the formation of gross haemorrhagic lesions in the pups lungs and focal necrosis of the liver (these necrotic lesions were found to contain tachyzoites). Parasites were also found in limb and ocular muscles of the pup (Dubey et al., 1988b). The lack of an acute infection caused by the inoculation of the homogenised pup tissues into the beagles (n=2), could have been due to insufficient numbers of viable parasites being present.

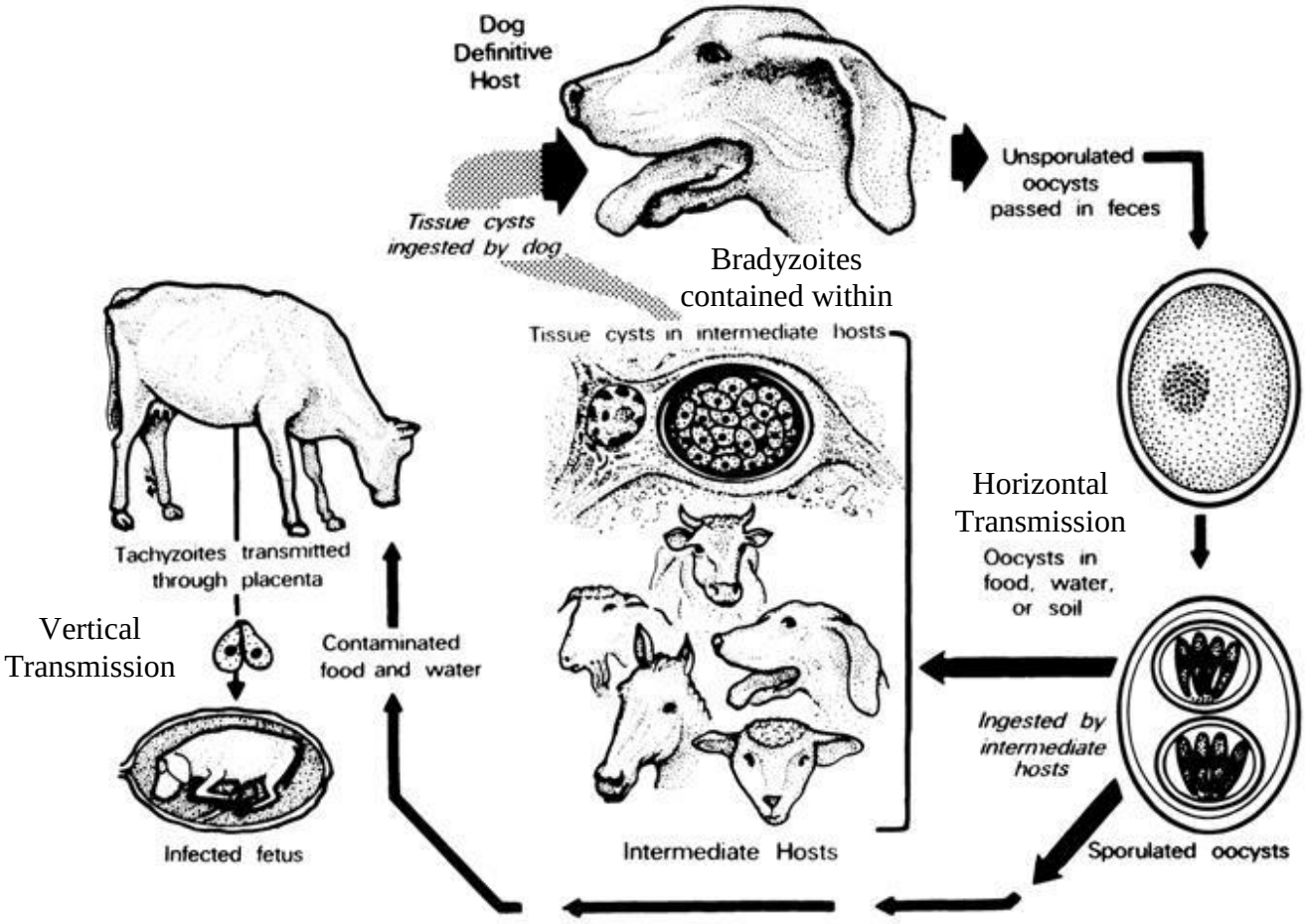
These initial experiments allowed Dubey and colleagues (1988b) to fulfil all of Koch's postulates, which were formulated by Robert Koch and Friedrich Loeffler and first published in 1890. The postulates are four criteria, which were designed to establish a causal relationship between a microbe and a clinical disease and are still used in microbiology today.

Koch's postulates are:

1. The microorganism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy organisms.

2. The microorganism must be isolated from a diseased organism and grown in pure culture.
3. The cultured microorganism should cause disease when introduced into a healthy recipient host.
4. The microorganism must be re-isolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.

Figure 1. Known life cycle of *Neospora caninum*



Copied and adapted from: *J.P. Dubey / Veterinary Parasitology 84 (1999) 349–367*

1.2 Known life cycle of *Neospora caninum*

For effective control strategies to be developed against *Neospora*, we need to understand the clinical implications of *Neospora* infections in important host species, e.g. cattle and dogs and the roles that these species play in the transmission of the disease.

A diagrammatic representation of the known life cycle of *Neospora* is illustrated in Figure 1. Following the ingestion of material containing parasite tissue cysts, dogs or other definitive hosts of *Neospora,* (see section 1.2.1) shed unsporulated oocysts. These oocysts sporulate and become infectious once in the environment, this can happen potentially in as little as 24 hours after excretion (Dubey et al., 2007). If these sporulated oocysts are ingested by intermediate hosts such as cattle (Gondim et al., 2004b) and sheep (O'Handley et al., 2002) (and many other animal species) through contaminated food or water, it can lead to the horizontal transmission of the parasite (see Figure 1). Once an intermediate host is infected, tissue cysts (containing bradyzoites) can form in the brain and other neural tissues, resulting in a chronic lifelong infection. However, should a primary infection occur whilst a host is pregnant, then the parasites can be transplacentally (vertically) transmitted to the foetus (see Figure 1), leading either to abortion, or the birth of a persistently (chronically) infected offspring (Dubey et al., 1989). Should the parasite induce an abortion and the placenta or aborted materials containing viable parasites be consumed by a naïve canid (Dijkstra et al., 2001), then oocysts can be shed and the life cycle can begin again. The oocysts could potentially

result in the epidemic spread of the parasite on a farm, resulting in an abortion storm (described in more detail in section 1.5.3)

1.2.1 Definitive hosts of *Neospora caninum*

Lindsay and Dubey (1990) demonstrated that *Neospora* tissue cysts were resistant to HCl-pepsin solution, suggesting that carnivorism may play a part in the life cycle of the parasite (Lindsay and Dubey, 1990). A definitive host of *Neospora* was confirmed in 1998, when a domestic dog (*Canis lupus familiaris*) was shown to shed oocysts following an experimental infection with *N. caninum* tissue cysts (McAllister et al., 1998). Since the discovery of the dog as a definitive host, other canid species including the Coyote (*Canis latrans*) (Gondim et al., 2004c), Australian dingo (*Canis lupus dingo*) (King et al., 2010) and Gray wolf (*Canis lupus*) (Dubey et al., 2011) have now also been demonstrated as natural definitive hosts for *Neospora caninum*.

Interestingly to date, the sexual stages of the *Neospora* have not been seen in any of the definitive host species currently identified. However it is known that in the closely related parasite species e.g. *Toxoplasma gondii,* schizogony (asexual reproduction) and gametogensis (sexual reproduction) both occur in the gut of a definitive host (a felid) (Hutchison et al., 1970). It is likely that the same processes of sexual reproduction (e.g. the formation of micro and macro gametes) occurs for *Neospora* in the gut epithelium cells of a canid, leading to the formation of unsporulated oocysts, which are then excreted in the faeces.

Neospora DNA has been found in a number of other carnivorous wildlife species including ferrets (*Mustela furo*), red foxes (*Vulpes vulpes*), European polecats (*Mustela putorius*), American mink (*Neovison vison*) and Eurasian badgers (*Meles meles*) (Bartley et al., 2013b). None of these other carnivorous species have been demonstrated as definitive hosts for *Neospora.* Though foxes (Schares et al., 2002b) and a number of mustelid species (ermine *(Mustela erminae*), weasels (*Mustela frenata*) and ferrets) (McAllister et al., 1999) have been examined experimentally to determine whether they shed *Neospora* oocysts following a primary infection, all of which were negative.

1.3 Known life stages of *Neospora caninum*

A better understanding of the life stages of the parasite and their roles in the transmission of the disease may affect the development of control strategies. For example a subunit vaccine containing unique antigens for all of the life cycle stages of *Neospora* would likely be more efficient than a vaccine that solely targets one life stage (i.e. the tachyzoite).

To date, the life cycle stages of *N. caninum* that have been identified are the tachyzoite, bradyzoite and the oocyst (containing sporocysts and sporozoites). All of these life cycle stages are known to be involved in the transmission of the parasite (Dubey et al., 2007), either between a definitive host and intermediate host (horizontal), or from a dam to a foetus (vertical) (see Figure 1).

1.3.1 Tachyzoite

The tachyzoites stage of *N. caninum* is motile, rapidly multiplying and actively invasive. Tachyzoites are ovoid, lunate or globular in shape and measure 3-7μm x 1.5μm depending on the stage of division (Dubey and Lindsay, 1993). Tachyzoites replicate by endodyogeny (asexual replication) into two daughter zoites, which are usually found within a parasitophorous vacuole located in the cytoplasm of the host cell. Tachyzoites contain all the organelles you would expect to find in a eukaryotic cell, including a nucleus, mitochondria, golgi complex, smooth and rough endoplasmic reticulum. However, tachyzoites also contain a number of additional organelles and structures (micronemes, rhoptries and dense granules) distinct to apicomlexan parasites, which are involved in host cell adhesion and invasion (Dubey and Lindsay, 1993) (Figure 2a). Tachyzoites have been identified in many cell types from naturally and experimentally infected animals, including neural cells, macrophages, fibroblasts, vascular endothelial cells, myocytes, renal tubular epithelial cells and hepatocytes (Dubey and Lindsay, 1993). This relatively broad tissue tropism allows the parasite to disseminate rapidly around a host, infecting many different organs and tissues (Dubey, 2003).

Tachyzoites are usually associated with active (acute) infections, during this early phase of an infection parasite DNA may be demonstrable by polymerase chain reaction (PCR) in the blood of infected animals (Dubey, 2003). The presence of parasites and parasite antigens in the circulatory system will lead to the generation both cell mediated and antibody responses against the parasite, which will be discussed in more detail in section

1.6. Tachyzoites have the ability to cross the blood–brain barrier, as well as invade other immune privileged sites such as the spinal cord (Dubey et al., 1988a) and optic nerves (Lindsay et al., 1996). Once in these tissues (sites of predilection), the parasite can differentiate (stage convert) into bradyzoites and form tissue cysts (section 1.3.2), causing a chronic (persistent) lifelong infection. In immune competent individuals the parasite remains sequestered in the brain (and other neurological tissues) causing little or no pathology (Anderson et al., 2000). However should the host become immunocompromised (i.e. during pregnancy), the bradyzoites can reactivate (recrudesce) and stage convert back into rapidly multiplying tachyzoites. An acute infection can ensue, the parasite may vertically transmit across the placenta, resulting in the abortion or the chronic *in utero* infection of the foetus (Dubey et al., 1990). In pregnant hosts the outcome of a *Neospora* infection depends on the stage of gestation and maturity of the foetal immune response (discussed in detail in 1.6.4).

1.3.2 Bradyzoites in tissue cysts

Tissue cysts containing bradyzoites are often round or oval and shape and can measure over 100µm in size (Figure 2b) (Dubey et al., 1988a, Lindsay et al., 1996). The bradyzoites contained within the tissues cysts are a similar size and shape as tachyzoites and contain the same apicomplexan specific organelles found within tachyzoites. However, bradyzoites are much slower replicating and are antigenically distinct to tachyzoites. Bradyzoites express a number of proteins and genes not seen to date in tachyzoites (i.e. SAG4, BSR4) (Fernandez-Garcia et al., 2006, Risco-Castillo et al.,

2007). Following the formation of tissue cysts, the parasite can persist in the host for life (Trees et al., 1999), as there are currently no treatments (chemotherapeutics) available that will kill the bradyzoites (Hemphill et al., 2015). Tissue cysts are predominantly found in either the brain or neural tissues (sites of predilection), though they can also form in many different tissues including liver (Dubey, 2003) and skeletal muscle (Peters et al., 2001). This ability of the parasite to infect multiple organs and tissues, means that effective biological control strategies are vital in limiting the spread of the disease (see section 1.5.6).

1.3.3 Oocysts

The oocyst is the most recently identified life cycle stage of *Neospora* and was described when the dog was confirmed as a definitive host (McAllister et al., 1998). Unsporulated oocysts are passed in the faeces of infected canids approximately 5 days after infection and can be passed for several months (Dubey et al., 2009). Once excreted into an aerobic environment, oocysts begin to sporulate in 1-3 days (Gondim et al., 2002). It is following sporulation that the oocysts become infectious and are then capable of causing parasite horizontal transmission (see Figure 1). Lindsay and colleagues (1999), clearly described the size (11.7 x 11.3µm), shape (spherical, subshperical) and internal structural arrangement of the sporulated oocysts. The sporocysts contained within the oocysts were ellipsoidal 8.4 x 6.1µm containing elongated sporozoites measuring 7.0 – 8.0µm x 2.0 – 3.0µm (Lindsay et al., 1999b).

It is likely that once consumed, the sporulated oocysts will excyst in the small intestines, with each oocyst releasing up to eight sporozoites (four from each of the two sporocysts). The sporozoites will then infect (parasitise) the intestinal epithelium cells and transform (stage convert) into tachyzoites. Following replication the *Neospora* tachyzoites will then spread via the circulatory system, resulting in an acute infection in the host.

The life cycle of *Neospora*, is clearly complex, with both horizontal and vertical transmission occurring during natural infections. Parasite stage conversions also occur in all the known life stages, in both definitive and intermediate hosts. All of these factors are important considerations that must be taken into account when trying to develop effective control strategies.

1.4 *In vitro* analysis of *Neospora caninum* tachyzoites

Since its first isolation into tissue culture (Dubey et al., 1988b), the *in vitro* growth of *N. caninum* parasites (tachyzoites) has proved an invaluable tool, not only for the propagation of the actively multiplying tachyzoite stage of the parasite, but also as a means of examining and comparing the rates of multiplication of parasite isolates.

The first *in vitro* studies to show differences in the *in vitro* growth (multiplication) rates of tachyzoites of different isolates was carried out by Innes and colleagues (1995). They

demonstrated that NC1 isolate tachyzoites replicated quicker than the Nc-Liv isolate tachyzoites, as measured by the incorporation of ^{3}H uracil (Innes et al., 1995). Another study has also shown differences in the multiplication rates of the NC1 and NC-Liv isolates, though interestingly during this second study NC-Liv was found to multiply faster than NC1 (Schock et al., 2001). It has been suggested that the longer tachyzoites are maintained (passaged) *in vitro*, the more they become "tissue culture adapted". However the findings of Schock and colleagues (2001) did not support this assertion. Schock and colleagues (2001) found that the parasite maintained for the longest period *in vitro* grew second fastest (NC1), while the second longest maintained parasite (Nc SweB1), was actually 5th slowest growing of 6 isolates tested, suggesting that the differences in the *in vitro* multiplication rates are genetic (Schock et al., 2001).

It has also been previously noted that prolonged *in vitro* cultivation of tachyzoites leads to a reduction in their *in vivo* pathogenicity (Long et al., 1998). Similar observations have been made for other parasites like *Babesia bovis* (Yunker et al., 1987), *Theileria annulata* (Preston et al., 2001) and *Leishmania* spp (Daneshvar et al., 2003), all of which have shown attenuation of *in vivo* virulence following prolonged *in vitro* culture. However 14 months of continuous culture of NC-Liv did not reduce its *in vivo* pathogenicity (Atkinson et al., 1999). While a study by Regidor-Cerrillo and colleagues (2011) has actually linked the increased invasion efficiencies of the NC-Spain 4 H and NC-Liv parasite isolates with increased virulence (Regidor-Cerrillo et al., 2011). It is clear that there are genetic differences between *Neospora* isolates (Campero et al., 2015, Regidor-Cerrillo et al., 2008). These differences will likely affect the rates of parasite

multiplication and *in vivo* virulence. However we still do not know what these differences are and if they can be exploited to help control the spread of the parasite.

1.5 Genetic diversity of *Neospora caninum*

1.5.1 *Neospora* Genome

DNA sequencing first appeared in the late 1970s and is now commonly referred to as Sanger sequencing, after one of the pioneering scientists working in the field, Frederick Sanger (Sanger et al., 1977). However it is since the advent of the second wave of sequencing technologies (also known as next generation sequencing (NGS), that high throughput, low cost sequencing has become available, which is capable of producing millions to billions of sequence reads per run (Blake, 2015), These NGS platforms include technologies such as 454 pyrosequencing, sequencing by synthesis (Solexa, illumina), sequencing by oligo ligation detection (SOLiD) and ion semiconductor sequencing (ion torrent) (Bennett et al., 2005, Margulies et al., 2005, Valouev et al., 2008).

Seminal work in the field of *Neospora* genomics was carried out by Reid and colleagues (2012), who were the first group to sequence the entire *Neospora* genome (using tachyzoites of the NC Liverpool (NCLiv) strain), Reid and colleagues (2012) demonstrated that the *Neospora* (NCLiv) genome is 61.0Mb in size and is divided into 14 chromosomes containing ~7500 predicted protein coding genes. The *Neospora*

genome is slightly smaller than that of *T. gondii* which is 63.0Mb, but is considerably larger than the genomes of *Plasmodium falciparum* which is only 23.3Mb long (Reid et al., 2012) and *Eimeria tenella* which is 51.8Mb, though interestingly *E. tennela*'s genome contains 8603 protein coding genes (Blake, 2015), which is ~1100 more genes than are found in *Neospora*.

As well as comparing the entire *Neospora* genome to other Apicomplexan parasites, work has also been carried out using random amplification of polymorphic DNA (RAPD) (Schock et al., 2001), as well as using mini and microsatellite technologies to examine the repetitive DNA sequences found in the *Neospora* genome (Goodswen et al., 2013). These repeated sequences are generally divided into three classes: highly repetitive, moderately repetitive and single (low) copy number sequences and provide an excellent source of DNA markers for investigating genetic diversity amongst parasites.

Some of the initial work on genetic diversity in *Neospora* was carried out by and colleagues (2004), who demonstrated variations in the internal transcribed spacer 1(ITS1) gene region between Brazilian, North American and European *Neospora* strains (Gondim et al., 2004a). More recently, multilocus genotyping has demonstrated extensive genetic diversity (polymorphisms) amongst laboratory maintained strains of *Neospora* (Al-Qassab et al., 2009, Regidor-Cerrillo et al., 2006).

Microsatelite technologies have not solely been used to examine the differences in laboratory strains, but have also demonstrated significant genetic differentiation between

field isolates of *Neospora*, where DNA was isolated from aborted bovine foetuses in Scotland, Germany and Spain (Regidor-Cerrillo et al., 2013). Basso and colleagues (2010) have also used microsatellites to examine epidemic abortion outbreaks in cattle on 5 farms in Germany, where for each of the epidemic outbreak a common microsatellite pattern prevailed. Each of the farms demonstrated a unique microsatellite profile, suggesting that on each farm the infection was through a common point source (Basso et al., 2010). Microsatellite markers were also used in one of the experiments described in this thesis (chapter 6), to demonstrate that a control dam used in the experimental infection during late gestation was infected prior to the study and not as a consequence of an inoculation error.

1.5.2 Transcriptomic analysis of *Neospora caninum*

Most of the transcriptomic analysis of *N. caninum* has been carried in comparison to *T. gondii*, where mRNA sequencing (or RNA*seq* as it is commonly known) has been used to compare the expression levels of protein coding genes of tachyzoites from the two closely related parasites. Some of the most dramatic findings of the RNA*seq* work include the observation that *Neospora* appears to have no functional gene for the rhoptry kinase ROP18 (a rhoptry neck protein). High levels of expression of ROP18 have been linked with parasite virulence in *T. gondii* (Reid et al., 2012). A suggested function for ROP18 in *T. gondii* virulence, is that ROP18 proteins inactivate host immunity related GTPases (IRGs) which would otherwise disrupt the PV, killing the parasites. *Neospora* is also entirely missing the locus encoding a number of other rhoptry neck proteins including ROP2A, ROP2B and ROP8. It has been hypothesised that this lack of ROP18

and a reduced *in vivo* virulence compared to *T. gondii* could in fact be an adaptation of *Neospora* allowing the efficient spread of the parasite without killing the recipient host (Reid et al., 2012).

Though *Neospora* appears deficient in a number of rhopty protein coding genes, it contains a much larger number of surface antigen (SAG) related sequences (SRS) genes with 227 compared to only 55 in *T. gondii*. Thought *Neospora* contains more SRS genes than *Toxoplasma*, in the tachyzoite stage, *Neospora* usually only expressed one gene at multigene locus, where *Toxoplasma* often expresses many (Reid et al., 2012). It has been suggested that this increased repertoire of SRS genes may actually have lead to the reduction in host range of *Neospora* compared to *T. gondii* (Reid et al., 2012) and that many of the SRS genes may be important in other developmental life cycle stages, such as bradyzoites.

Though genomic differences have been described between strains of *Neospora*, at a phenotypic (Goodswen et al., 2013) and antigenic (Schock et al., 2001) level *Neospora* strains are extremely similar. The use of –omics data (genomics, transcriptomics and proteomics) should pinpoint the reasons for these differences, however to date there have not been any in depth –omics comparisons of strains of *Neospora* and is an area that requires significant further investigation.

Figure 2. *Neospora caninum* tachyzoites (Panel A) and a tissue cyst containing bradyzoites in the brain (Panel B)

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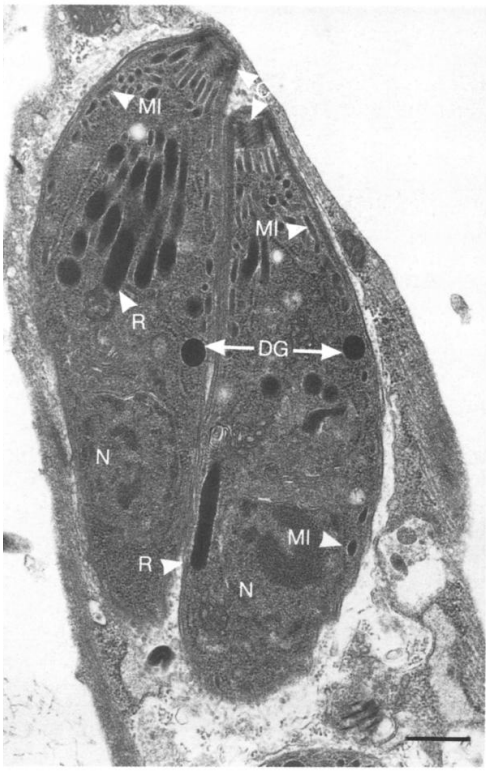
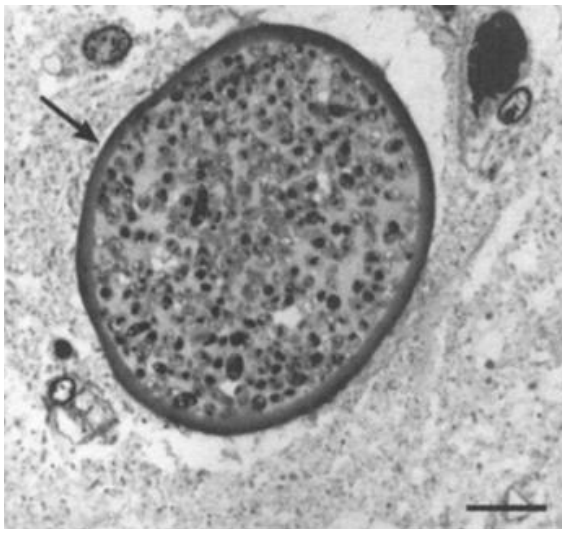
A



B



A, Note several rhoptries (R) with electron-dense contents anterior to the nucleus and one rhoptry posterior to the nucleus. Also, note the conoid (C), micronemes (MI), dense granules (DG), nucleus (N) and mitochondria (MT) in the tachyzoites. Scale bars = 0.5 μm. Copied from: *J.P. Dubey and D.S. Lindsay Parasitol Today 9 (1993), 452-458*

1.6 Bovine neosporosis

1.6.1 Economic impact and importance of bovine neosporosis

The true economic impact of bovine neosporosis is difficult to calculate, as often the only directly attributable effect of *N. caninum* infection in cattle is the abortion of the foetus. The direct economic impact of an aborted foetus is the immediate loss of the value of the aborted animal, whether that is a dairy replacement animal or a beef animal destined for the food chain. However, there are also the added costs associated with having to service the dam again, leading to an increased calving interval. Two studies in Canada have demonstrated that animals that are seropositive for *N. caninum* have poorer reproductive performance than seronegative animals (Waldner et al., 2001a) and consequently have longer calving intervals (Waldner, 2005). A number of studies in England have all sited that a major reason for culling in dairy herds is poor levels of fertility amongst animals (Esslemont and Kossaibati, 1997, Whitaker et al., 2000, Whitaker et al., 2004). While other studies have demonstrated that *Neospora* positive animals were 1.6-1.73 times more likely to be culled than seronegative animals (Bartels et al., 2006, Thurmond and Hietala, 1996).

Experimental evidence has shown that a primary *Neospora* infection during early pregnancy in cattle can result in foetal resorption (Maley et al., 2006). The economic impact of *Neospora* induced early foetal death and resorption in cattle is very difficult to estimate, as no prevalence data is available. How often foetal resorption occurs in natural infections with *N. caninum* is unknown, but it is likely to equate to a

considerable if unquantifiable economic loss to the cattle industry. Many of the animals suffering early foetal death and resorption may appear as having an increased time to conception, hence an increased calving interval, or present as being permanently infertile.

Amongst dairy herds the profitability of an animal increases with age (Esslemont and Kossaibati, 1997). Culling an animal before its fourth lactation, may in fact lead to a net loss for a farmer, as the net milk sales against feed costs in the first three lactations are not considered large enough to be profitable (Esslemont and Kossaibati, 1997). *Neospora* seropositivity in dairy animals alone has not been linked to a significant decrease in milk production compared to seronegative animals (Bartels et al., 2006). However, a study in Canada showed that seropositive cows in herds with abortions attributable to *N. caninum* infection produced significantly less milk than seronegative cows (Hobson et al., 2002). The apparent lack of consistency in milk data might be caused by a number of factors including, differences in the virulence of the parasite strains infecting the cattle, farm husbandry practices and the geographical regions where the data was collected. The individual experimental design, selection criteria of animals and farms, as well as the methods of data collection and analysis may also affect the experimental results.

All of the economic effects of bovine neosporosis described above, will have a profound negative impact on the saleable value of infected animals (Trees et al., 1999). Leading to an overall reduction in the value of breeding stock, especially given the fact that animals

remain infected for life and can transmit the parasite to their progeny (potentially over several successive generations).

A recent article attempted to quantify the economic looses to both the dairy and beef industries worldwide. To do this, data was collected from papers published in Australia, New Zealand, Canada, Mexico, United States of America, Argentina, Brazil, the Netherlands, Spain and the United Kingdom, describing natural outbreaks of bovine neosporosis (Reichel et al., 2012). These data sets have estimated the total median economic impact of *N. caninum* in both beef and dairy industries is approximately US $1.298 billion per annum. The diary industry incurred the biggest annual losses at approximately US$842 million, while the beef industries annual sustained losses are approximately US $455 million. The last comprehensive survey in England and Wales attributed approximately 12.5% of abortions to neosporosis (Davison et al., 1999b), Reichel and colleagues estimated that these losses in the UK dairy industry were in the region of US$27 million per annum (Reichel et al., 2012).

1.6.2 Routes of parasite transmission of *Neospora caninum*

Any strategies to control *Neospora* transmission must take into account the complex nature of the life cycle of the parasite (Figure 1). Natural *Neospora* infections are known to occur in both definitive (Dubey et al., 1988a) and intermediate hosts (Dubey et al., 1989) and can involve any of the known life cycle stages of the parasite (Anderson et al., 2000). So targeting one life cycle stage or one host species may not limit the spread

of the disease, a broader approach involving all economically important species is required.

1.6.3 Natural routes of *Neospora* infection in cattle

There are two major natural routes of infection with *N. caninum*, these are: the vertical (transplacental transmission) route, where an infected dam passes the infection directly to her foetus (Dubey et al., 2006), or the horizontal (post natal) route, where naïve animals (juveniles and adults) become infected through the ingestion of food or water contaminated with *Neospora* oocysts (Figure 1) (Dubey, 1999b). Horizontal transmission can also occur through the ingestion of tissues / organs infected with bradyzoites, or aborted placental / foetal tissues containing tachyzoites, though infection in this manner is most likely to occur in carnivorous or omnivorous animals, such as dogs and other canids (Dubey, 2003).

Transplacental (vertical) transmission can occur as a consequence of infection in two different ways. Endogenous transplacental transmission (En TT) occurs as a result of the recrudescence (reactivation) of a persistent (chronic) infection in a dam, leading to an active acute infection and the vertical transmission of the parasite to the foetus. Exogenous transplacental transmission (Ex TT) occurs if a dam becomes infected for the first time during pregnancy, through the ingestion of sporulated oocysts (Figure 1) (Trees and Williams, 2005, Williams et al., 2009) and may lead to abortion (Gondim et al., 2004c), or the chronic *in utero* infection of the foetus.

Other natural routes of infection that have also been suggested, these include the venereal spread of the parasite through tachyzoite contamination in semen (Ortega-Mora et al., 2003). Placentophagia (the consumption of a infected placenta by another intermediate host) has also been suggested as another natural route of infection (Modry et al., 2001). However these routes of transmission have never been demonstrated in naturally occurring cases of bovine neosporosis and are unlikely to result in an infection. The parasite stage most likely to be involved would be the tachyzoite, which would be unlikely to survive long enough in such hostile environments as the gastric tract and uterus to cause an active acute infection in a healthy host.

1.6.4 Experimental routes of *Neospora* infection in cattle

Two routes of experimental infection have been extensively used in cattle, these are the intravenous (iv) or subcutaneous (sc) routes of inoculation (Macaldowie et al., 2004, Williams et al., 2000, Williams et al., 2009). The results from experimental studies comparing iv and sc challenges suggest that the iv route of inoculation leads to more severe clinical disease than a sc challenge (Macaldowie et al., 2004). This increased disease severity may in part be due to the haemotogenous nature of the iv challenge. Parasites rapidly enter the circulatory system causing placental and foetal damage before an appropriate immune response can be mounted (Macaldowie et al., 2004, Williams et al., 2000). Compared to the much slower sc route of infection, where the parasite must first migrate through tissues before reaching the circulatory system; during which time

the host will be starting to mount innate, cell mediated and humoral immune responses against the parasite.

Other experimental routes of *Neospora* infection in cattle have also been examined; these include conjunctival (de Yaniz et al., 2007), intrauterine (Serrano-Martinez et al., 2007) and intramuscular (Andrianarivo et al., 2001) challenges with tachyzoites. Studies have also used oral infections of *Neospora* oocysts in cattle (De Marez et al., 1999, McCann et al., 2007). However, none of these latter methods of administering the parasite are routinely used in large scale experimental infections in cattle.

1.6.5 Epidemiology of *Neospora caninum* infections in cattle

The main clinical symptom associated with bovine neosporosis is abortion, with *Neospora* being the most commonly diagnosed cause of infectious bovine abortion in the UK (Davison et al., 1999a). It has been estimated that approximately 13% of abortions in cattle in the UK and 20% of abortions in the USA are attributable to *Neospora* (Davison et al., 1999a, Dubey, 2003). *Neospora* infections are wide spread, in the Netherlands seropositive animals were found in 78% of dairy herds tested (Wouda et al., 1999a) and in 73% of dairy herds tested in Quebec, Canada (Pare et al., 1998). In healthy non-pregnant animals clinical symptoms rarely present following a *Neospora* infection (Wouda, 2000). Though natural clinical cases of neosporosis are uncommon, they have been reported in cattle (Dubey and Rommel, 1992) as well as in sheep and goats (Eleni et al., 2004, Moreno et al., 2012). The symptoms usually present in neonatal animals and can include paralysis, muscle atrophy and hind limb hyperextension (Barber

and Trees, 1996, Dubey and Lindsay, 1996). These clinical symptoms are associated with severe neurological damage, leading to early neonatal mortality (Dubey and Lindsay, 1993). Usually in experimentally challenged cattle, the only overt clinical symptoms seen during an acute *Neospora* infection are a short term febrile response (increased rectal temperature) and mild swelling (oedema) may be observed around sites of parasite inoculation (Maley et al., 2006, Maley et al., 2003b).

Naturally occurring *Neospora*-associated bovine abortions have been described as having either an epidemic (abortion storms due to point source exposures) or an endemic pattern (recrudescence of chronic infections) (Thurmond and Hietala, 1997).

1.6.6 Epidemic *Neospora* abortions in cattle

Neospora induced epidemic abortion storms, are associated with primary *Neospora* infections of herds (both naïve calves and adult animals) *en mass*. Usually through exposure (ingestion) to sporulated oocysts, shed by a definitive host (i.e. a dog) (Figure 1), leading to the exogenous transplacental transmission (Ex TT) of the parasite. A number of studies have examined natural point source outbreaks of *Neospora* (mass Ex TT events), that have induced abortions. These studies have defined an epidemic (abortion storm) as being when 10 - 15% of all susceptible animals at risk in a herd, abort within a 4 - 8 week period (Moen et al., 1998, Schares et al., 2002a, Wouda et al., 1999a).

1.6.7 Endemic *Neospora* abortions in cattle

Endogenous transplacental transmission (En TT) is likely to be the major route of infection within herds of cattle considered to have endemic abortion problems. Though EnTT is unlikely to cause the mass abortion storms that can be seen following Ex TT events, it is likely to result in long term persistent problems on farms. This is in part due to the efficiency with which *Neospora* can be vertically transmitted, it has been suggested that between 80 – 95% of seropositive cattle will congenitally infect their offspring (Davison et al., 1999a, Pare et al., 1996). There is extensive evidence from both naturally and experimentally infected cattle, to support the association between infection status and risk of abortion. A study looking at congenitally infected heifers demonstrated that during their first pregnancy, *Neospora* infected cattle were 7.4 times more likely to abort than age matched non-infected controls (Thurmond and Hietala, 1997). Data from the Netherlands demonstrated a 2–3 times increased likelihood of foetopathy in *N. caninum* infected animals, compared to uninfected animals (Moen et al., 1998). While, a study looking at over 1000 randomly selected cows in England and Wales demonstrated that *Neospora* infected animals were 3.5 times more likely to abort than seronegative animals (Davison et al., 1999b). Though the results from these studies were collected from a wide geographical area, they show a broadly similar outcome, with *N. caninum* infected cattle being approximately 3-4 times more likely to abort than negative animals. The data from these studies also suggests that the likelihood of abortion or foetopathy is higher in primiparous animals, compared to multigravida dams that have successfully carried more than one pregnancy to term.

1.6.8 Parasite strain virulence of *Neospora caninum*

The effect of the "strain" on the outcome of an infection is known to be important factor across the study of almost all infectious agents. But what exactly is a strain. McKenzie *et al*., (2008) in a review of *Plasmodium* species described "strains" as autonomous, stable biological entities that are distinguishable from each other by clinical virulence, epidemiological or other features such as antigenic properties, drug resistance and ability to infect (McKenzie et al., 2008). This is compared to an "isolate" which McKenzie and colleagues describe as a sample of parasites which are not necessarily genetically homogeneous and have been collected from a naturally infected host on one single occasion.

Though not all of the criteria used by McKenzie and colleagues to categorise *Plasmodium* parasites are true for *Neospora,* a number of them are. The NC1 parasites that are used throughout the experiments described in this thesis (Chapters 2-7) would fulfil the criteria of both a strain and an isolate. The NC1 parasites have been shown using both RFLP and Microsatelite technologies (Al-Qassab et al., 2009, Gondim et al., 2004a) to be clearly distinguishable from other strains of *Neospora*. While the NC1 tachyzoites themselves were isolated from a single naturally infected host (a Labrador Retriever pup) (Dubey et al., 1988a) fulfilling the criteria of McKenzie and colleagues to also be considered as an isolate!

Some isolates of *N. caninum* have been shown to be more pathogenic in cattle than others. Experimental challenges of cattle with the NC1 and Nc-Liv isolates have been

shown repeatedly to be highly pathogenic, causing abortion and foetopathy (Maley et al., 2003b, Williams et al., 2000). While other isolates such as NcSweB1 (Quinn et al., 2002) and Nc-Spain 1H (Rojo-Montejo et al., 2009b) appear to be less virulent in cattle. These naturally occurring differences in virulence make the choice of parasite challenge strain crucial for experimental infections. The less pathogenic strains may not be suitable for use as challenge parasites, in vaccination/challenge models. If the challenge parasites naturally cause little or no pathology, a vaccine may appear more effective, than if a more virulent isolate of *Neospora* had been used as the challenge.

1.6.9 Use of experimental models to examine the effect of the infectious dose of *Neospora caninum* parasites on the clinical outcome of disease

The challenge dose of parasites is critical to the clinical outcome of a *N. caninum* infection. Work on cattle, carried out by Maley and colleagues (2003) demonstrated that a subcutaneous (sc) challenge with $1x10^7$ live tachyzoites (NC1 isolate) caused milder disease symptoms than a sc challenge with $5x10^8$ tachyzoites (Maley et al., 2003b).

Similar findings have been observed using mouse models, where an increased inocula size resulted in greater lesion severity and an increased cerebral parasite loads (Collantes-Fernandez et al., 2006, Long et al., 1998). However, a recent study by Arranz-Solis and colleagues (2015) demonstrated similar levels of neonatal mortality in mouse pups in groups of dams receiving $1x10^5$ tachyzoites compared to those receiving 100 tachyzoites (NC-Spain 7 strain). These results suggest, that in many experiments an excessive and highly unrealistic number of parasites are being administered (Arranz-

Solis et al., 2015). This excessive dose results in high levels of parasitaemia, which may make identifying immune-reactive antigens and antigen combinations difficult, as the host immune response may be overwhelmed before a protective response can be generated. However we must use caution when comparing these data sets, as the experiments employed different model species, used different parasite strains, doses and routes of inoculation.

Over recent years there have been calls to try and standardise experimental *Neospora* infections conditions in cattle, to allow easier comparisons of data from different research groups and experiments. If a standard dose was used then comparisons regarding the pathogenicity of isolates could be more easily made (Benavides et al., 2014). However, a standard route of administration would also have to be employed, as discussed in section 1.6.2.2, the route of inoculation also clearly plays a critical role in the clinical outcome of the infection.

1.6.10 Methods used in the routine diagnosis of bovine neosporosis

Detection of *Neospora* infections in cattle has routinely been conducted using one of two methods. One is the microscopic examination of aborted foetal tissues, following post mortem examination. Using microscopy allows a differential diagnosis to be reached, if parasites are observed in lesions in the foetal tissues (Thilsted and Dubey, 1989). Alternatively serological analysis can be used to detect anti-*Neospora* antibodies (Dubey et al., 1988b), as there are no currently no reliable *ante mortem* polymerase

chain reaction (PCR) tests to detect a parasitaemia in the brain or blood naturally infected cattle.

Commonly used serological tests for bovine neosporosis are based on either whole tachyzoites for immunofluorescent antibody tests (IFAT) (Dubey et al., 1988b), or antigen generated from tissue culture derived tachyzoites, for enzyme linked immunosorbent assays (ELISA) (Bjorkman et al., 1994). While an IgG avidity ELISA will allow the serological discrimination between acutely and chronically infected animals, as the avidity of IgG antibodies increase over time. So the higher the antibodies avidity the longer an animal has been infected (Bjorkman et al., 1999). Other methods such as the *Neospora* (direct) agglutination test (NAT) (Romand et al., 1998) and the latex agglutination test (LAT) (Moraveji et al., 2012) have also been developed for *Neospora* diagnosis. However the method of choice for large scale serological screening is the *Neospora* tachyzoite antigen ELISA.

This reliance on whole tachyzoites / tachyzoite antigens for use in diagnostics may have led to an under estimation of the levels of infection within herds of cattle, as chronically (persistently) infected animals may appear seronegative when tested against *Neospora* tachyzoites antigens (Conrad et al., 1993). In a recent study by Mazuz and colleagues (2014), approximately 10% of dams that tested serologically negative for *Neospora* gave birth to persistently infected offspring (Mazuz et al., 2014). Chronically infected animals are only likely to have a detectable parasitaemia (circulating tachyzoites) if they are undergoing parasite recrudescence. This usually only occurs when bradyzoites stage

convert back to tachyzoites, usually following host immunomodulation (i.e. pregnancy, immune suppression). In immunologically normal hosts (i.e. non-pregnant, immunocompetent) bradyzoites elicit minimal inflammatory reaction (Anderson et al., 2000). Humoral responses against *Neospora* bradyzoites are highly variable (Aguado-Martinez et al., 2008) and the levels of anti-*Neospora* bradyzoite antibodies will depend on the intensity and duration of antigen specific exposure during parasite reactivation (Guido et al., 2016). Recombinant versions of a number of bradyzoite specific proteins including SAG4 (Fernandez-Garcia et al., 2006) and BSR4 have been created (Risco-Castillo et al., 2007). These recombinant bradyzoites antigens could be used to try and serologically detect chronic cases of neosporosis, in animals that were previously thought to be uninfected using a conventional tachyzoite antigen ELISA. However it is unclear if the sensitivity of assays using these antigens would be sufficient to accurately detect low anti-bradyzoite antibody responses in naturally infected animals. One method that may improve the rates of serological detection would be to use a combination of tachyzoite and bradyzoite antigens (Aguado-Martinez et al., 2008).

1.6.11 Current strategies for the control of bovine neosporosis

The most effective strategy to control bovine neosporosis would be a vaccine capable of inhibiting the vertical transmission of the parasite, this topic is discussed in detail in section 1.8. There are also currently no licensed chemotherapeutics for use in cattle that will kill the chronic tissue cyst stages of the parasite (Dubey et al., 2007). In the absence of a vaccine or chemotherapeutics, many of the strategies for controlling bovine

neosporosis are aimed at reducing the risks of primary infection (abortion storms) through good animal husbandry practices.

Current husbandry strategies used to control bovine neosporosis include; reducing canine faecal contamination in and around cattle feed bins, water sources and pastures. Limiting the access of dogs (in particular young naïve dogs) to carcasses and aborted bovine material (foetal and placental) is also advised (Cavalcante et al., 2011). While feeding dogs raw meat should also be avoided where possible, Especially following the discovery of *Neospora* tissue cysts in bovine skeletal tissues from naturally infected animals (Peters et al., 2001). Work by Kramer and colleagues (2004) demonstrated that feeding dogs raw bovine material was a significant risk factor associated with seropositivity in canine neosporosis (Kramer et al., 2004). Rodent control and restricting the natural hunting behaviour of dogs may also prevent them from ingesting naturally infected wildlife reservoir host species (e.g. mice and rats) (Ferroglio et al., 2007) and thus prevent canine infection (Gondim, 2006). Work in the Netherlands demonstrated that there is a strong correlation between seropositivity of *N. caninum* antibodies in farm dogs and a high prevalence of anti-*Neospora* antibodies in the cattle (Wouda et al., 1999b).

Farmers may also choose not to use seropositive animals for breeding replacement stock, to try and reduce the incidence of En TT. Embryonic transfer has also been used to produce seronegative calves. Taking embryos from *Neospora* positive animals and implanting them into *Neospora* negative recipient animals resulted in the total

abrogation of parasite vertical transmission (Baillargeon et al., 2001, Landmann et al., 2002). However, this method is likely to be very expensive and the economic benefit would probably only be seen in high value pedigree herds.

The most drastic control measure that may be implemented on a farm would be the removal of all known infected cows and their offspring, also referred to as the test and cull method. However, this is only practical in low prevalence herds, as culling may have huge economic implications for the farmer (Wouda, 2000). Also, if there is under reporting of cases of bovine neosporosis, due to false negative serological tests, then infected animals may remain within herds, defeating the purpose of culling or removing known infected animals.

1.7 Host - parasite relationship during bovine neosporosis

A clear understanding of the involvement of all aspects of a protective immune response (innate, humoral and cell mediated) is required for any control strategy to be successful in inhibiting the transmission of *Neospora*. Knowledge relating to the importance of the type (Th1 or Th2), magnitude and location of these responses will also be critical in developing an effective vaccine.

To be able to accurately study the immunological responses being produced by a host following an infection, species specific (or highly cross reactive) reagents are required.

Sadly until recently this is an area where immunological studies in cattle have struggled. There is less of a market for bovine and ovine immunological reagents than for the more widely funded human and rodent immunology. One of the first commercially produced bovine reagents was the BOVIGAM ELISA kit, which was produced by CSL in 1988 and is used to detect the production of interferon gamma (IFN-γ) by cattle following an infection. The BOVIGAM kit is still commonly used in many countries today to test for bovine Tuberculosis (*Mycobacterium bovis*) infections (Wood and Jones, 2001). Since the development of the IFN-γ ELISA a number of other tests have been developed for other key cytokines including interleukin (IL)-4 (Hope et al., 2005), IL-10 (Kwong et al., 2002) and IL-12 (Hope et al., 2002), and tumour necrosis factor alpha (TNF-α) (Kwong et al., 2010), most of these assays are used to measure immune responses in pregnant dams and their foetuses in this thesis (Chapters 6-7). As well as ELISA for the detection of cytokines, antibodies are also available to detect a wide range of surface markers on immune cells, and key immunological components like toll like receptors (Kwong et al., 2011). Advances in molecular biology along with NGS and the first whole genome sequencing (WGS) of *Bos taurus* in 2004 (Zimin et al., 2012) have enabled the development of many other reagents such as qPCR primers and probes, that allow researchers to determine changes in the level of gene expression of key immunological components like TLR's (Chang et al., 2009).Immune responses of non pregnant cattle to a primary infection with *Neospora caninum*

Early work examining the immune responses against *Neospora* were conducted by Innes and colleagues (1995), who demonstrated that interferon-γ (IFN-γ) could significantly (*p*

= 0.02) reduce *in vitro* rates of parasite multiplication (Innes et al., 1995). While Marks and colleagues (1998) demonstrated the importance of $CD4^+$ T-cells and the production of IFN-γ in response to a primary *Neospora* infection in cattle (Marks et al., 1998). Flow cytometric assays have shown that though $CD4^+$ T-cells are the predominant producers of IFN-γ in response to *Neospora*, both $CD8^+$ T-cells and Natural killer (NK) cells also contribute to IFN-γ production at early stages following a *Neospora* infection (Klevar et al., 2007). Work by Staska and colleagues (2003) showed that $CD4^+$ T-cells not only work in a T-helper role, but also have a cytotoxic effect on *Neospora* infected autologous target cells (Staska et al., 2003). These results combined demonstrate the importance of $CD4^+$ T-cells and IFN-γ in generating a protective Th1 type immune response against *Neospora*.

Natural killer cells harvested from *Neospora* infected cattle have been used to examine the involvement of the innate immune responses during early *Neospora* infections. Live and heat inactivated *Neospora* tachyzoites triggered interleukin 2 (IL-2) activated bovine NK cells into producing IFN-γ independently of the cytokine interleukin 12 (IL-12). Though the presence of IL-12 increased the magnitude of IFN-γ response seen in activated NK cells, which also showed increased cytotoxic activity when co-cultured with *Neospora* infected autologous fibroblasts (Boysen et al., 2006). Natural killer cells are important effector cells in the initiation and priming of an adaptive immune response against *Neospora*. Boysen and colleagues (2006) findings suggest that though an immune response can be initiated against *Neospora* in an IL-12 independent manner, IL-

12 is clearly a potent activation factor for the innate immune response against the parasite.

Interestingly both of the studies by Boysen and colleagues (2006) and Klevar and colleagues (2007) demonstrated that bovine NK cells failed to respond when stimulated with soluble *Neospora* tachyzoite antigens. This suggests that either intact tachyzoites or non-soluble NK cell stimulating tachyzoite antigens are required for NK cell triggering (Boysen et al., 2006, Klevar et al., 2007). This lack of NK cell activation by an incomplete repertoire of tachyzoite proteins may partially explain why *Neospora* subunit vaccines have failed to initiate a fully protective immune response against the parasite in cattle (discussed in more detail in section 1.8.2). It is likely that the activation process of a cellular response is highly complex, involving interactions between many facets of the immune response.

1.7.1 Maternal immune responses to *Neospora caninum* in cattle during different stages of gestation

The maternal immune responses against *Neospora* in pregnant cattle have been extensively studied, using both experimental and natural infection systems. These studies have demonstrated the importance of lymphoproliferative cell mediated responses and the production of IFN-γ in the protective immunity against *Neospora* (Andrianarivo et al., 2001, Rosbottom et al., 2011, Williams et al., 2000).

During early pregnancy the location of a maternal immune response is critical to determining disease outcome following either a primary infection, or during the recrudescence of a chronic *Neospora* infection. Strong infiltrations of $CD3^+$, $CD4^+$ T-cells, gamma-delta (γδ) T-cells and NK cells at the maternal placenta were linked to foetal death (Canton et al., 2014b, Maley et al., 2006). These infiltrating cells were also associated with increases in the expression of the Th1 type cytokines IL-12, IFN-γ and tumour necrosis factor α (TNF-α) at the placental interface (Canton et al., 2014a, Rosbottom et al., 2008), causing severe immune mediated pathology and foetal death (Macaldowie et al., 2004). These results were supported by Gibney and colleagues (2008), who showed that a challenge with *Neospora* tachyzoites during early pregnancy in cattle caused extensive focal epithelial necrosis and the infiltration of maternal mononuclear cells at the placenta, which was associated with foetal deaths (Gibney et al., 2008). While work by Flynn and Marshall (2011) demonstrated that macrophages from *Neospora* infected cattle were producing IL-17A as well as IFN-γ, and suggested that a combination of these two pro-inflammatory cytokines could be contributing to the immunopathology seen at the maternal foetal interface during *Neospora* infections in cattle (Flynn and Marshall, 2011).

At mid gestation, maternal lymphoproliferative and IFN-γ responses against *Neospora* antigens have been detected in both the peripheral blood mononuclear cells (PBMC) and peripheral lymph nodes of experimentally challenged pregnant cattle. However these immune responses were not sufficient to stop parasite vertical transmission, but may have limited the severity of foetal infection (Bartley et al., 2004). Responses to the

mitogen concanavalin A, as well as antigen specific proliferative responses and IFN-γ production have been shown to be down regulated at mid gestation, irrespective of infection status of the dams (Innes et al., 2001). Similar observations were made by Almeria and colleagues (2003) who showed a suppression of maternal responses in PBMC at around day 126 (week 18) of gestation (Almeria et al., 2003). The immune responsiveness of dams has been shown to return by late gestation (Innes et al., 2001). This modulation of the maternal immune response is likely to be caused by the endocrine system. Levels of the hormone progesterone increase steadily in pregnant cattle from early to mid-gestation (Pope et al., 1969). While prostaglandin E2 which is also produced during pregnancy, is known to suppress T-cell proliferation and inhibit interleukin -2 (IL-2) production (Chouaib et al., 1985), thus biasing a cell mediated immune (CMI) response towards a Th2 type response (Kalinski et al., 1997). Trophoblast and decidual cells have also been shown to suppress immune responses (Raghupathy, 1997), through the release of decidua-associated suppressor signals (Clark et al., 1984). While some lymphocytes can produce progesterone-induced blocking factor (PIFB). The presence of PIFB along with IL-4 and IL-10, will also bias an immune response away from Th1, towards a Th2 type response (Raghupathy, 1997). This immunomodulation (towards a Th2 type response) could explain why rates of vertical transmission increase as gestation progresses, as it may allow parasite recrudescence to occur, leading to foetal infections (see Figure 3). This may also explain why many *Neospora* associated bovine abortions occur at around day 150 of gestation (Dubey, 1999a). If during a chronic infection large numbers of parasites recrudesce and cross the placenta, they may overwhelm the developing foetal immune responses and

result in an abortion. However a lower parasite challenge could be controlled by the foetus, resulting in chronic infection but no foetal death, reiterating the importance of the infectious parasite dose.

The experimental evidence examining *Neospora* infections during late pregnancy in cattle is limited. The available data suggests that primary infections at this stage of pregnancy are often associated with high rates of vertical transmission, but low rates of foetal mortality. Andrianarivo and colleagues (2001) demonstrated vertical transmission and foetal infection in 5/5 dams challenged with *Neospora* during late pregnancy (days 219-231 of gestation) (Andrianarivo et al., 2001). While Benavides and colleagues (2012), observed vertical transmissionin in all infected animals from 28 days post challenge (day 238 of gestation) (Benavides et al., 2012). Previous observations have also shown that following a challenge with *Neospora* at late gestation foetal viability was associated minimal placental necrosis, mild inflammatory infiltration and reduced levels of IFN-γ expression (Gibney et al., 2008, Rosbottom et al., 2008). These findings were supported by the work of Canton and colleagues (2014a and 2014b). Where is was shown that the lack of foetal death was likely due in part to the mild inflammatory cellular infiltration at the maternal placenta (Canton et al., 2014b), with scarce production of pro inflammatory cytokines (i.e. IFN-γ) (Canton et al., 2014a). This lack of immunopathology is likely due to the Th2 biased environment at the placenta, leading to a reduction (suppression) of the harmful pro-inflammatory responses (Raghupathy, 1997). However, the main factor that will reduce the rates of foetal death is the

increasing maturity of the foetuses immune response against *Neospora* as gestation progresses.

1.7.2 Ontogeny of the foetal bovine immune system

Due to the syndesmochorial (synepitheliochorial) nature of the ruminant placenta, under normal circumstances no maternal immune factors including immune cells, antibodies and cytokines can cross the maternal-foetal interface. This means that any immune responses seen *in utero* are as a direct result of foetal infection. Implantation of the bovine foetus begins to occur around day 19 of gestation (MacIntyre et al., 2002). In a normal healthy bovine foetus, lymphoid development is seen from day 42 of gestation, while the spleen is structurally present by day 60 of gestation (Schultz et al., 1973). Monocytes and T-cells have been shown in large numbers at 3 months of gestation in bovine foetal thymus and spleen samples. At the same gestational period (approx. 90 days) low numbers of B-cells were also identified. The lower concentration of B-cells is as expected, as B-cells predominantly proliferate and differentiate in an antigen dependent manner (Senogles et al., 1979). Experimental data from cattle clearly shows that as gestational age increases, the risk of foetal death associated with a primary *Neospora* infection decreases (Andrianarivo et al., 2001). However as gestation progresses the chance of the parasite being vertically transmitted to the foetus increases (Figure 3) (Benavides et al., 2012).

1.7.3 Development of the bovine foetal immune responses against *Neospora caninum*

As described in section 1.6.3, in cattle the structure of a foetal immune system is in place from as early as 42 – 60 days of gestation (Schultz et al., 1973). Considerable research has focused on whether the foetus is capable of mounting an effective immune response against a *Neospora* infection and if so, at what stage of gestation does the foetal immune response become protective? Though immune cells and lymphoid tissues are present during the first trimester of pregnancy, the cells do not appear capable of mounting protective antigen specific immune responses. Should vertical transmission occur during the early stages of pregnancy the result will likely be foetal death (Andrianarivo et al., 2001, Rosbottom et al., 2008).

By mid gestation the bovine foetal immune response is more fully developed and has been shown to be capable of mounting antigen specific immune responses from about 131 – 154 days of gestation (Almeria et al., 2003). These responses are predominated by $CD4^+$ T-cells and IFN-γ production (Almeria et al., 2003, Bartley et al., 2004, Rojo-Montejo et al., 2009a). By mid gestation the bovine foetuses are also capable of mounting humoral immune responses against *Neospora*, which is predominated by IgG1 isotype antibodies (Andrianarivo et al., 2001).

There is currently a lack of experimental data examining the bovine foetal immune responses against *Neospora* during late gestation, most experiments have focused on either the maternal immune responses (Rosbottom et al., 2007) or the immunopathology

at the placenta (Gibney et al., 2008). The available data shows that by the third trimester of pregnancy bovine foetuses are able produce strong humoral responses, with anti-*Neospora* antibody titres of ≥1:20,000 (tested by IFAT) and CMI responses against *Neospora,* including lymphocyte proliferation and IFN-γ production in PBMC, mesenteric lymph node (LN), prescapular LN and spleen (Andrianarivo et al., 2001).

1.7.4 The effect of host genetic diversity in cattle on immunological responses against *Neospora caninum*

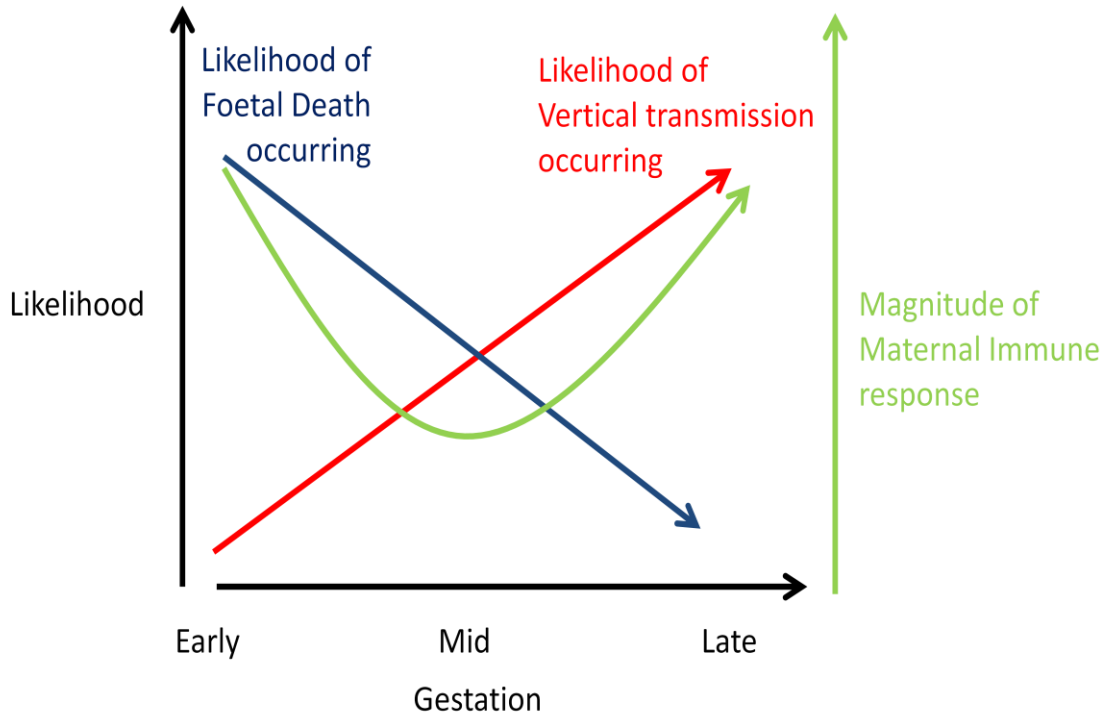
An area of research that has not been extensively explored in the field of bovine neosporosis is the role host genetic diversity plays in resistance to infection; in particular the role of the major histocompatability complex (MHC) class II. The genes encoding the MHC II are known to be highly polymorphic, producing large numbers of distinct alleles, for example the DRB3*2703 and 2707 alleles are associated with resistance to a number of disease including bovine Leukemia virus (BLV) (Schwab et al., 2009) In *Neospora* infected cattle the DRB3*1001 allele was associated with pregnancy loss, while DRB3*2703 was found only in dams that gave birth to live healthy calves (Schwab et al., 2009).

Neospora infected Cattle with disparate MHC haplotypes have been shown to develop parasite specific $CD4^+$ T-cells which killed autologous infected cells, but not heterologous infected targets, this is consistent with MHC restricted $CD4^+$ mediated

parasite killing (Staska et al., 2003). Further work by Staska and colleagues (2005) has demonstrated that MHC class II heterozygous Holstien cattle infected with *Neospora* all demonstrated IFN-γ secretion by $CD4^+$ cytotoxic T-lymphocytes (CTL) directed against *Neospora* NcSRS2 peptides. Suggesting that the cattle can respond against *Neospora* irrespective of MHC haplotype (Staska et al., 2005). These findings were further supported by the work of Baszler and colleagues (2008), who also demonstrated that *Neospora* infected cattle with disparate MHC haplotypes all responded to NcSRS2 peptides with lymphoproliferative and IFN-γ responses (Baszler et al., 2008).

Though there is still quite limited data regarding the impact of MHC haplotypes on protection against *Neospora* infections, it is clear that the MHC class II plays an important role in the protective immunity and needs to be considered when developing future vaccine strategies against bovine neosporosis.

Figure 3. Graphical representation of the relationship between the gestational age of the bovine foetus, the magnitude of the maternal immune response and the likelihood of foetal death or the vertical transmission of *Neospora* occurring



1.8 Investigating the host – parasite relationship of *Neospora caninum* using experimental mouse models

Mice are known to be intermediate hosts for *Neospora*, with naturally infected animals having been found in numerous countries around the world (Dubey, 2003). However, mice are probably most important as experimental model systems for examining the disease dynamics, immunology and pathology relating to *Neospora* infections. Experimental mouse models offer an excellent opportunity to study the host–parasite interaction in a cost effective manner, prior to committing to large expensive experimental cattle studies. We must however be careful when extrapolating the immunological findings from mice and applying them to larger animals (i.e. ruminants), although it is an obvious statement "a mouse is not a cow"; murine immunology as well as placental biology (haemochorial) differs greatly from that of ruminants (syndesmochorial). Thus the host/parasite interactions, particularly around the placenta may also be different. Though there are clear differences in immunology and biology between mice and cattle. The ease with which mice can be handled, the short duration of murine pregnancies (19 - 20 days) (Lopez-Perez et al., 2008) and the number reagents available to study murine immunology, make mouse models an excellent starting point for many *in vivo* experiments.

As with all *in vivo* experimentation, consideration must also be given to host strain and parasite isolate choices, as these can have an impact on the outcome of infection. Some of the differences in clinical manifestations that are observed in the host, may be a result

of natural genetic resistance to *Neospora* infection. For example, work using microarray analysis to compare BALB/c and Qs mice, demonstrated clear transcriptional differences in levels of gene expression between the two breeds of mice. These differences were observed 6 hours after a subcutaneous challenge with either NC Liverpool or NC-Nowra tachyzoites (Ellis et al., 2010). Compared to the susceptible BALB/c mice, the resistant Qs mice showed upregulation of the genes regulating the Jak-STAT signalling pathway (Stat2 and Stat3), which is involved in B-cell development and the regulation of IL-10 and IL-12 expression. This upregulation of early immune response genes in the Qs mice will likely lead to improved priming of the immune response, which may help reduce the parasite dissemination and the associated pathology.

1.8.1 Host-parasite interactions of *Neospora caninum* in non pregnant experimental mouse models

Non pregnant mice offer an opportunity to examine the immunological responses during acute and chronic (cerebral) neosporosis, in animals which develop a clear symptomatology, that is easily characterised within a matter of days following infection (Collantes-Fernandez et al., 2006, Rojo-Montejo et al., 2012).

Mice also offer excellent opportunities for investigating *Neospora* dissemination, allowing easy comparisons of the tissue trophisms of different parasite isolates to be made. Work by Collantes-Fernandez and colleagues (2006) showed that during acute *Neospora* infections, parasites can be detected in the blood and numerous other organs,

while during chronic infections that parasites are usually limited to the brain (Collantes-Fernandez et al., 2006). Similar findings were reported by Pereira Garcia-Melo and colleagues (2010), where a mouse model was used to examine the pathogenicity of a number of *Neospora* isolates (from asymptomatic calves). Parasite DNA was found in the blood and lungs of mice during acute infections, then only in the brains of chronically infected mice (Pereira Garcia-Melo et al., 2010). These data indicate that we must sample appropriate organs and tissues, depending on whether acute or chronic infections are being investigated. For example should a parasite isolate be being examined for its ability to persist long term following an infection, then the brain and central nervous system (CNS) should be the preferred sampling choices. However during acute phase dissemination studies, the blood, and visceral organs would be better options to find parasites / parasite DNA (Collantes-Fernandez et al., 2006).

Many aspects of the murine immune response against *Neospora* have been examined and these finding have often been used to steer and inform immunological and vaccine research in ruminants. Some of the earliest work using a mouse model clearly demonstrated the importance of IFN-γ and IL-12 (Baszler et al., 1999, Khan et al., 1997, Long et al., 1998) in a protective immune response against an acute infection with *Neospora* tachyzoites. While Ritter and colleagues (2002) demonstrated that the adoptive transfer of IFN-γ producing immune cells to a IFN-γ / genetic knockout mouse could significantly lengthen survival, following a challenge with *Neospora* tachyzoites (Ritter et al., 2002). Nishikawa and colleagues (2003) demonstrated that resistance to an acute *Neospora* infection in wild type BALB/c mice was associated with increased

production of both IFN-γ and IL-4, compared to susceptible mice (Nishikawa et al., 2003) Though the Th1 associated cytokines are critical in a protective immune response, a balance must also be found with the Th2 types cytokines, in particular IL-4 and IL-10. However an immune response that is overly-biased toward a Th2 type response can also be detrimental. This was the case in BALB/c mice immunised with soluble tachyzoites antigen, then challenged with *Neospora* tachyzoites, where an increase in IL-4 production was correlated with exacerbated encephalitis and neurological disease in the vaccinated mice, compared to the unvaccinated / challenged controls (Baszler et al., 2000). It appears that immunisation with tachyzoite antigens led to the generation of an inappropriate overly-biased Th2-type response, which was detrimental to the survival of the mice following challenge. These data are important considerations for vaccine development, as a vaccine needs to produce a balanced, appropriate and proportionate immune response against the parasite, which will not result in either pathology or chronic infections.

The importance of the humoral immune response during a *Neospora* infection was confirmed using a mouse model. B-cell deficient C57BL/6 μMT mice were shown to be more highly susceptible to infection with *Neospora* tachyzoites than wild type C57BL/6 mice. This susceptibility resulted in greater parasite dissemination, leading to a lethal outcome of infection. The μMT mice's susceptibility was not just associated with the deficiency in B-cells and the absence of anti-*Neospora* antibodies, but was also manifested through decreased antigen specific proliferation of the splenocytes and reduced IFN-γ production (Eperon et al., 1999). This lack of immunological

responsiveness of the splenocytes is likely due to poor immune priming caused by the deficient B-cells. The importance of immune priming during *Neospora* infections was demonstrated using flow cytometic analysis of $CD69^+$ activated B-cells, from *Neospora* infected BALB/c mice. The activated B-cells demonstrated increased surface expression of the molecules CD80 and CD86, which are known to interact with CD28 on the surface of T-cells, leading to T-cell activation, and the production of various interleukins (Teixeira et al., 2005).

Antibodies directed against the *Neospora* tachyzoite surface protein NcSRS2 have been generated in mice. These antibodies inhibited the *in vitro* attachment and invasion of parasites to ovine foetal trophoblast cells. It is hypothesized that the inhibition may have been achieved by the antibodies binding to and inducing conformational changes in the NcSRS2 protein on the tachyzoites surface, which in turn meant that the protein was unable to recognise host cell surface molecules. Alternatively the antibodies may have just physically blocked the interactions between the parasites and the corresponding host cell surface proteins (Haldorson et al., 2006).

Mouse models have also been used to demonstrate the importance of the innate immune response during primary *Neospora* infections. Work by Mineo and colleagues (2009 and 2010) showed that myeloid differentiation factor 88 (MyD88) and toll like receptor-2 (TLR-2) are involved in the initial immune recognition of *Neospora* parasites in mice, leading to the generation of a protective effector immune response (Mineo et al., 2009, Mineo et al., 2010). Upregulation of MyD88 is associated with IL-12 production and

increased IFN-γ responses in $CD4^+$, $CD8^+$ T-cells and NK cells (Mineo et al., 2009), while TLR-2 is involved in antigen presenting cell (APC) activation and T-cell proliferation (Mineo et al., 2010). All of the processes controlled by the innate immune response are critical to inducing (activating) a protective immune response against *Neospora*. TLR-$2^{-/-}$ C57BL/6 Knockout mice that lack of these innate processes are known to show increased susceptibility to *Neospora* infection, compared to wild type C57BL/6 mice (Mineo et al., 2009, Mineo et al., 2010).

When examining the host - parasite relationship in non pregnant animals it is important to remember that immune factors that are potentially important in controlling infection in non pregnant animals may be affected by the immunomodulation that occurs during pregnancy and may no longer have the same protective effect. This could however be overcome if a protective immune response could be established before a host becomes pregnant.

1.8.2 Host–parasite interactions of *Neospora caninum* using experimental pregnant mouse models

Experimental infections of *Neospora* using pregnant mouse models have been comprehensively examined, looking at both acutely (Long and Baszler, 1996, Lopez-Perez et al., 2008) and chronically infected animals (Jimenez-Ruiz et al., 2013, Omata et al., 2004, Rettigner et al., 2004).

Mouse models have been used to examine differences in the virulence of parasite isolates and the transmission dynamics of *Neospora,* in pregnant mice (Pereira Garcia-Melo et al., 2010, Rojo-Montejo et al., 2009b). However Regidor-Cerrillo and colleagues (2010) carried out the most comprehensive study, where the rates of vertical transmission in mice, were examined using 10 *Neospora* isolates. Following an acute infection, differences in the rates of neonatal mortality (from 32 – 95%) and vertical transmission (from 50 – 100%) were identified between the isolates (Regidor-Cerrillo et al., 2010). These results indicate clear variations in the pathogenicity (virulence) and transmission efficiency of isolates of *Neospora*.

Like in cattle, a number of studies in mice have shown that the timing of acute infection during pregnancy affects the clinical outcome. Acute infections of mice on day 7 of gestation not only lead to increased levels of neonatal mortality, but also lead to the retardation of normal development in surviving pups (Lopez-Perez et al., 2010).

Infection during mid pregnancy (days 7-12 of gestation) resulted in higher levels of neonatal mortality compared to infection during mid-late pregnancy (days 12-14 of gestation) (Liddell et al., 1999, Lopez-Perez et al., 2008). There is likely to be greater disease severity at early-mid gestation compare to mid-late gestation, because during the early stages of pregnancy (day 7 of gestation) the placenta is still developing and the embryo has only just implanted (on approx 4-5 days of gestation), therefore any parasites reaching the placenta are likely to cause more severe damage leading to transplacental infection, while in later gestation the placenta is fully formed and the

embryo is more developed, creating a more effective barrier to parasite invasion (Lopez-Perez et al., 2010).

Collantes-Fernandez and colleagues (2012) made an interesting discovery that could explain how *Neospora* parasites bypass the haematochorial placenta of mice, when they showed that *Neospora* tachyzoites exploit natural cellular traficking pathways, by infecting dendritic cells (DCs) to help cross cell barriers (i.e. transplacental transmit) (Collantes-Fernandez et al., 2012). The importance of this cellular traffic in ruminants is however unknown, as under normal conditions a syndesmochorial placentation will not allow the transfer of maternal immune cells. However, this form of cellular trafficking would aid parasites in rapidly disseminating around a host and reaching the placenta. Allowing the parasite more time to establish and replicate, increasing the parasites chances of vertically transmitting, before an immune response can be initiated.

In mice chronically infected with *Neospora,* efficient vertical transmission of the parasite from dam to offspring has been shown in the first generation of breeding (Omata et al., 2004). However by the 2^{nd} and 3^{rd} generations of breeding from chronically *Neospora* infected mice, almost no vertical transmission was observed (Jimenez-Ruiz et al., 2013, Rettigner et al., 2004). This would suggest that a natural immunity is developing in the mice against the parasite across successive generations. This data would suggest that only primigravida animals should be used in experiments when trying to compare rates of vertical transmission.

The results from experimental mouse models support the findings seen in cattle, indicating that strong Th1 type immune responses, along with the innate and humoral responses are all required to successfully control *Neospora* infections and inhibit the vertical transmission of the parasite.

1.9 Prospects for developing a vaccination strategy to control the spread of *Neospora caninum*

As with many other protozoan parasites, the development of a vaccine is an important goal in the area of *Neospora* research. Experimental evidence from cattle shows that animals can be protected against vertical transmission (Innes et al., 2001) and that prior exposure to *Neospora* decreased the chances of abortion compared to a primary infection with the parasite (McAllister et al., 2000). So a vaccine that mimics the immune responses generated by live *Neospora* should create a solid immunity against the parasite.

1.9.1 Live parasite vaccines tested against acute *Neospora caninum* infections using experimental mouse models

There is a considerable body of data examining the effects of live vaccine formulations used to protect mice against experimental infections with *Neospora*. Lindsay and colleagues (1998) produced temperature sensitive isolates of *Neospora* that protected

against a subsequent lethal challenge. However, the attenuation was not stable and the temperature sensitive isolates reverted to a virulent wild type state when re-cultured at 37^{o}C (Lindsay et al., 1999a). While inoculation with γ-irradiated tachyzoites also protected against a lethal primary challenge with *Neospora* (Ramamoorthy et al., 2006). However the efficacy of the temperature sensitive tachyzoites and γ-irradiated parasites were never tested in a pregnant mouse model. A live transgenic NC1 strain of *Neospora* tachyzoites (Nc-1 SAG4(c)2.1) which constitutively expressed the bradyzoite specific gene SAG4, has also been tested in pregnant mice. Vaccination with Nc-1 SAG4(c)2.1 produced significant reductions in levels of vertical transmission in challenged animals compared to unvaccinated controls. However the transgenic strain of *Neospora* still induced chronic infections in mice and has not been tested in ruminants (Marugan-Hernandez et al., 2011b). Rojo-Montejo and colleagues (2012), tested an experimental vaccine consisting of the naturally low virulence Nc-Spain 1H parasites in mice, the vaccination was shown to completely abrogate cerebral infection in all vaccinated animals challenged with *Neospora* Nc-Liv tachyzoites (Rojo-Montejo et al., 2012). The evidence clearly shows that live *Neospora* vaccines are effective at producing a protective immune response, when tested in mice.

1.9.2 Live vaccines tested in cattle against natural and experimental infections with *Neospora caninum*

A number of research groups have also examined the efficacy of live parasite vaccines using pregnant cattle. Innes and colleagues (2001) demonstrated that prior exposure to *Neospora* induced protection against the vertical transmission of the parasite using an

experimental cattle model. Cattle were vaccinated with *Neospora* tachyzoites prior to pregnancy, challenged at mid gestation and then monitored through to birth. Calves born to vaccinated dams were negative for anti-*Neospora* antibodies and PCR negative for parasite DNA. These results suggest that the immune responses generated in the dams prior to pregnancy protected against the vertical transmission of the parasite following a challenge during mid pregnancy (Innes et al., 2001). In a study by Rojo-Montejo and colleagues (2013) Nc-Spain 1H parasites failed to completely protect against the vertical transmission of *Neospora* when tested in cattle. However vaccination with Nc-Spain 1H parasites did lead to a reduction in the rates of vertical transmission and increased neonatal survival, following a mid-gestation challenge with Nc-Liv tachyzoites compare to the unvaccinated animals (Rojo-Montejo et al., 2013). Webber and colleagues (2013) have also shown a reduction of vertical transmission by between 70-90% (depending on the route of vaccination). Heifers were vaccinated either iv or sc with Nc-Nowra parasites before pregnancy, then challenged with virulent *Neospora*Nc-S197 tachyzoites on day 139 of gestation. One dam that was vaccinated (iv) with Nc-Nowra but not challenged aborted a *Neospora* PCR positive foetus. Indicating that the attenuation of Nc-Nowra is incomplete and there is still the capacity for a reversion to virulence (Weber et al., 2013). A field trial of vaccinations with the *Neospora*NcIs491 isolate, led to a 10% reduction in abortions in vaccinated compared to non-vaccinated cattle, but appeared to have no effect on inhibiting vertical transmission in naturally infected animals (Mazuz et al., 2015). Hecker and colleagues (2015) showed that animals vaccinated with live NC-6 *Neospora* parasites prior to pregnancy then challenged on day 70 of gestation showed minimal placental infiltrations of $CD4^+$, $CD8^+$, γδ T-cells and

macrophages, and very low levels of expression of the cytokines IFN-γ, IL-4, IL-10, IL-12 and TNF-α (Hecker et al., 2015), though no data on rates of vertical transmission were presented in this experiment. The results of Hecker and colleagues (2015) agree with the finding of Innes and colleagues (2001) and suggest that the immune responses generated by the dams prior to pregnancy have successfully limited the spread of the parasites; meaning that very few parasites are reaching the placenta, resulting in a very mild inflammatory response.

The need for more research into developing a live vaccine to control bovine neosporosis was raised by Reichel and colleagues (2015) in a recent letter to the journal "Vaccine". The letter urged pharmaceutical companies, veterinarians, farmers and researchers alike to invest in the development of a live vaccine to treat bovine neosporosis, as a live vaccine will likely be more efficacious than either killed or subunit vaccines (Reichel et al., 2015).

1.9.3 Killed vaccines tested against experimental infections with *Neospora caninum* in mice

Killed vaccines have had mixed success in producing protective immune responses against *Neospora* infections in mice. Inoculation with crude Nc-Nowra tachyzoite antigen, reduced the incidence of vertical transmission, however this inhibition was much less successful compared to a vaccination with live Nc-Nowra parasites (Miller et al., 2005). Heat killed tachyzoites have also been shown to induce lower levels of IFN-γ production in splenocytes and bone marrow derived dendritic cells than a comparable

dose of live parasites, suggesting poorer immune priming by the heat killed tachyzoites, compared to the live parasites (Feng et al., 2010). Vaccination with heat killed tachyzoites has also actually led to an increase in host susceptibility to *Neospora* in mice (Baszler et al., 2000).

1.9.4 Killed vaccines tested against natural and experimental infections with *Neospora caninum* in ruminants

In a field trial, the killed *Neospora* tachyzoite vaccine (Neoguard®) was administered to cattle naturally infected with *Neospora*, vaccination led to a reduction in rates of abortion by 9% compared to control animals. However no data was given comparing rates of vertical transmission from these animals (Romero et al., 2004). A more recent study looking at Neoguard® actually showed an increased risk of abortions in vaccinated cattle, compared to the unvaccinated controls (Weston et al., 2012). The findings of Weston and colleagues (2012) agree with the previous findings from mouse studies, in that using killed vaccines can lead to increased host susceptibility to *Neospora* infections. This lack of protection induced by killed vaccines could in part be explained by the observations made by Boysen (2006) and Klevar (2007), who suggested that killed vaccines lack key immune stimulatory molecules that are needed to generate a protective immune response.

Crude tachyzoite antigens have also been used to try and vaccinate sheep against *Neospora* infections. These experiments showed a slight decrease in rates of abortion in vaccinated animals compare to the controls, but vertical transmission was still seen in

both vaccinated and challenged / control animals (Jenkins et al., 2004b, O'Handley et al., 2003).

Though live vaccines have many efficacious advantages over killed vaccines (i.e. they actually work), they are more problematic to produce. Pharmaceutical companies are less likely to want to produce live vaccines compared to killed / subunit vaccines as they are comparatively expensive to manufacture, usually require a cold chain for storage, have a short shelf life and have the capacity to revert to virulence.

1.9.5 Testing of subunit vaccines against *Neospora caninum* in experimental mouse models

Recombinant subunit vaccines, both monovalent and polyvalent (multivalent) antigen cocktails have been tested for their ability to create a protective immune response against primary infections and the vertical transmission of *Neospora*. The target antigens for these subunit vaccines are often either parasite surface antigens, like *Neospora* surface antigen NcSRS2 (Pinitkiatisakul et al., 2007), surface antigen 1 (SAG1) (Cannas et al., 2003a) or antigens relating to the molecules involved in cellular invasion, like rhoptory protein 1 (ROP1) (Debache et al., 2009), microneme (MIC) proteins, MIC1 (Ramamoorthy et al., 2007a), MIC3 (Cannas et al., 2003b) and the dense granule (GRA) proteins GRA2, GRA6 (Ramamoorthy et al., 2007b) and GRA7 (Jimenez-Ruiz et al., 2012) or the bradyzoite specific surface antigens like BSR4 (Marugan-Hernandez et al., 2011a) and SAG4 (Jimenez-Ruiz et al., 2012).

Many monovalent antigens fail to elicit protective responses when administered alone, most need to be give as polyvalent antigen cocktails before any meaningful responses are seen. For example the antigens SAG4, BSR4 and SRS9 when used independently did not offer protection (Jimenez-Ruiz et al., 2012), likewise no protection against vertical transmission was observed using Nc MIC3-R (Monney et al., 2012) and NcMIC1, NcMIC3 and NcROP2 (Monney et al., 2013) independently. However a number of antigen cocktail formulations have shown promise in limiting or completely inhibiting vertical transmission of *Neospora* in pregnant mouse experiments (Haldorson et al., 2005, Jenkins et al., 2004a, Liddell et al., 2003, Nishikawa et al., 2001).

To date, the only subunit vaccine cocktail tested in ruminants, failed to produce positive results. A vaccination combining rNcSAG1, rNcHSP20, rNcGRA7 using immune stimulating complexes (ISCOMS) adjuvant failed to prevent foetal infection in cattle challenged on day 70 of gestation (Hecker et al., 2014). Cattle vaccinated with this cocktail of recombinant antigens (rNcSAG1, rNcHSP20, rNcGRA7) and challenged on day 70 of gestation showed severe placental infiltrations of $CD4^+$, $CD8^+$, γδ T-cells and macrophages, and increased expression of the cytokines IFN-γ, IL-4, IL-10, IL-12 and TNF-α. Compared to animals vaccinated with live parasites that showed minimal infiltration of immune cells and low levels of cytokine expression at the placenta. These results suggest that the recombinant antigens are were unable to stop the parasites reaching the maternal-foetal interface, resulting in immune mediated pathology (Hecker et al., 2015).

1.9.6 Appropriate adjuvant selection for use with subunit and killed vaccines against *Neospora caninum*

Appropriate adjuvant selection is likely to be critical to the success of a subunit / killed vaccine. Many adjuvants have been tested with limited success, these include Immunmax (Vertex®) used with crude tachyzoite antigen preparations in pregnant sheep (Jenkins et al., 2004b, O'Handley et al., 2003). Havlogen (Intervet®) which was used in conjunction with the Neoguard® vaccine (Weston et al., 2012). Immune stimulating complexes (ISCOMS) was used with a multivalent subunit vaccine but failed to stop foetal infection, following a challenge on day 70 of gestation in pregnant cattle (Hecker et al., 2014). However vaccines using Polygen^{TM} (MVP Laboratories) and Freunds have stimulated immune responses comparable to infection with live parasites (Andrianarivo et al., 1999, Baszler et al., 2008, Staska et al., 2005). These two adjuvants (Polygen and Freunds) may have been more successful as they induce a Th1 type response. However these formulations have not been tested for their efficacy in preventing vertical transmission and abortion in pregnant cattle.

To date the only method that appears to clearly protect against the vertical transmission of *Neospora* in cattle is the exposure of animals to live parasites prior to pregnancy. Prior exposure to the *Neospora* tachyzoites leads to the generation of a long lasting protective immune response, capable of stopping vertical transmission (Innes et al., 2001). However the possibility of a reversion to virulence of *Neospora* tachyzoites is probably the biggest safety concern that is limiting the development of a live vaccine against bovine neosporosis.

1.10 Aims

The aims of the experiments included in this thesis were:

- To determine whether long term *in vitro* cultivation (tissue culture) of *Neospora caninum* tachyzoites produced an attenuation of virulence when tested in an experimental mouse model.
- To determine whether tissue culture attenuated *Neospora caninum* tachyzoites could protect naïve animals against a known lethal challenge with the parasite.
- To determine if there were differences in the immunological responses of hosts following an inoculation with either tissue culture attenuated or wild type virulent *Neospora caninum* tachyzoites.
- Examine the maternal and foetal immune responses in cattle challenged with *Neospora caninum* tachyzoites at different stages of gestation to help identify key components of a protective immune response
- Examine the immunological responses of *Neospora caninum* infected cattle to liquid chromatographically fractionated water soluble tachyzoite antigen, with the purpose of identifying cell populations responding to immunoreactive peptides, which could potentially be used as subunit vaccine candidates.

2 Long term passage of tachyzoites in tissue culture can attenuate virulence of *Neospora caninum in vivo*

P. M Bartley, S Wright, J. Sales, F. Chianini, D. Buxton and E.A. Innes

(2006)

Parasitology

2.1 Manuscripts main hypotheses

The main hypotheses hat were tested in this manuscript are:

1. Does an extended period of *in vitro* cultivation attenuate the *in vivo* virulence of *Neospora caninum* tachyzoites when using a non pregnant mouse model?
2. Are both attenuated and virulent parasites capable of causing clinical disease in an experimental mouse model?
3. Is the severity of clinical disease caused by the *Neospora* tachyzoites dose dependent?
4. Are there differences in the *in vitro* growth characteristics of the attenuated and virulent tachyzoites?

The results from this series of experiments clearly demonstrated that the tachyzoites maintained for a longer period in tissue culture (referred to as the attenuated parasites) showed decreased *in vivo* virulence, compared to the wild type (virulent) tachyzoites. This attenuation was manifest through reduced levels of morbidity and mortality in inoculated animals. However both wild type (virulent) and attenuated parasites were still capable of causing clinical disease in mice, which increased in severity when the parasite dose was increased. Both the virulent and attenuated parasites were capable of causing not only acute but also chronic infections in the host, as was evident through the presence of tissue cysts containing baradyzoites, which were found in the brains of mice receiving either virulent or attenuated parasites. However the incidence of tissue cysts was reduced in the mice receiving the attenuated parasites compared to those receiving

the virulent parasites. Using the incorporation of ^{3}H Uracil as a measure of *in vitro* intracellular multiplication, the attenuated tachyzoites were shown to multiply faster than the virulent parasites between 6, 12, 24, 48, 72 and 96 hours, suggesting the attenuated parasites have become more tissue culture adapted than the virulent tachyzoites.

Though clear evidence of attenuation was demonstrated during this study, the attenuation is incomplete as the attenuated parasites are still able to cause chronic infections (form tissue cysts) and the mechanisms by which the parasites have become attenuated is still unclear. Further comparisons of the virulent and attenuated parasites are required to determine processes involved in attenuation. One method that could yield useful and interesting results would be RNAseq (transciptomic) comparison of the parasites, this would highlight any differences in the expression of protein coding genes and may also identify proteins which could be exploited in helping to control *Neospora*.

The numbers of parasites used for each infectious dose are also too high. A reduced number of parasites would have improved the survival rates / times of the animals, This would have allowed for more rigorous screening and comparisons of the histopathological changes in the brains of animals receiving either the attenuated or the virulent parasites at later time points, which were unavailable during this current experiment due to the early culling of many animals.

2.2 Author contributions

PMB, SW, DB and EAI were involved in all aspects of the experimental design and planning of the experiments. This included the morbidity scoring system which was created in accordance with the guidelines agreed by the UK Home Office. All experiments were approved by Moredun Research Institutes experimental ethics committee.

PMB and SW inoculated all the mice and were responsible for making clinical observations, daily morbidity scoring, weighing of individual mice and the culling of animals when needed. At post mortem examination PMB and SW collected all tissue samples for PCR and histological analysis and blood samples for serological screening.

PMB maintained the Vero cells and *Neospora* tachyzoites in tissue culture, enumerated the cells and tachyzoites using a Neubauer haemocytometer and prepared all of the inocula (Vero cells and tachyzoites) used during the experiments and was responsible for performing all *in vitro* growth rate experiments comparing the virulent and attenuated parasites. PMB extracted the DNA from all of the tissue samples examined and performed all ITS1 PCR analysis. PMB collated all of the data including the *in vitro* growth rates, morbidity, mouse weight, and mortality data and prepared all of the graphs and tables presented in the manuscript.

SW separated serum from clotted blood samples and performed all serological testing (IgM and IgG) by IFAT, while FC and DB performed all histological and immunohistological analysis and provided the images presented in the manuscript.

JS performed all statistical analysis on the mean morbidity score, mouse weight, group morbidity and IFAT data.

PMB, SW and EAI drafted the original manuscript with contributions from all other authors.

Long-term passage of tachyzoites in tissue culture can attenuate virulence of *Neospora caninum in vivo*

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SUMMARY

To determine whether prolonged *in vitro* passage would result in attenuation of virulence *in vivo*, *Neospora caninum* tachyzoites were passaged for different lengths of time *in vitro* and compared for their ability to cause disease in mice. Groups of Balb/c mice were inoculated intraperitoneally with 5×10^6 or 1×10^7 of low-passage or high-passage *N. caninum* tachyzoites. The mice were monitored for changes in their demeanour and body weight, and were culled when severe clinical symptoms of murine neosporosis were observed. Mice inoculated with the high-passage parasites survived longer ($P<0{\cdot}05$), and showed fewer clinical symptoms of murine neosporosis, compared to the mice receiving the low-passage parasites. The parasite was detected in the brains of inoculated mice using immunohistochemistry and ITS1 PCR. Tissue cysts containing parasites were seen in mice inoculated with both low-passage and high-passage parasites. When the *in vitro* growth rates of the parasites were compared, the high-passage parasites initially multiplied more rapidly ($P<0{\cdot}001$) than the low-passage parasites, suggesting that the high-passage parasites had become more adapted to tissue culture. These results would suggest that it is possible to attenuate the virulence of *N. caninum* tachyzoites in mice through prolonged *in vitro* passage.

Key words: *Neospora caninum*, *in vitro* culture, attenuation *in vivo*.

INTRODUCTION

Neospora caninum is an apicomplexan parasite, first described by Bjerkås *et al*. in 1984 and isolated and named by Dubey *et al*. (1988). *Neospora caninum* has a worldwide distribution and is a major cause of reproductive failure in cattle (Innes *et al*. 2001). Considerable economic losses are attributed to *N. caninum* in the farming industry, including the costs of still birth and neonatal mortality, increased calving interval resulting from early foetal death, increased culling, reduced milk production and reduced value of breeding stock (Trees *et al*. 1999; Dubey, 2003). There are currently no suitable chemotherapeutic agents to prevent transplacental transmission or to eliminate the parasite in cattle, making the development of an effective vaccine highly desirable. Epidemiological evidence indicates that persistently infected animals are less likely to abort following a point-source exposure than previously naïve animals, suggesting that cattle may develop some protective immunity against the disease (McAllister *et al*. 2000). Due to the intracellular nature of the parasite, it is expected that cell-mediated immunity (CMI), involving lymphoproliferative responses and the production of the cytokine interferon gamma (IFN-γ) will have a major protective role (Innes *et al*. 1995; Khan *et al*. 1997; Baszler *et al*. 1999). Eperon *et al*. (1999) demonstrated the importance of B-cells (and presumably antibodies) in controlling *Neospora* infections in mice. Although the role of antibodies in a protective immune response has still to be determined, a likely function would be in controlling the spread of extracellular stages of the parasite (Innes *et al*. 2002). Recent experimental studies in cattle have demonstrated that inoculation with *N. caninum* prior to pregnancy can protect against a second challenge administered at mid-gestation; transplacental transmission only occurred in the naïve control animals (Innes *et al*. 2001). In addition Williams *et al*. (2003) showed that seropositive cattle naturally infected with *N. caninum* were protected against abortion when experimentally challenged in early gestation. These two studies indicate vaccination may be a feasible option for the control of bovine neosporosis.

Neospora caninum infections can be established in mice, providing a convenient experimental model with which to test potential vaccination strategies. Live vaccines are more likely to induce appropriate CMI responses in host animals (Innes *et al*. 2002). Protective immunity against acute infection has

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been induced in mice using live attenuated, temperature-sensitive mutant strains of *N. caninum* (Lindsay *et al*. 1999) and sublethal doses of live *N. caninum* tachyzoites (Lundén *et al*. 2002). Virus-vectored vaccines including canine herpes virus (CHV) and vaccinia virus expressing the NcSRS2 *N. caninum* protein have also induced protective immune responses in mice (Nishikawa *et al*. 2000, 2001).

Attenuation of parasite virulence has been successfully applied in the development of live vaccines against other protozoan parasites. *Leishmania major* and *Leishmania mexicana* have been attenuated by repeated *in vitro* passage in the presence of gentamicin, and both attenuated strains of *Leishmania* induced significant protection in mice challenged with wild-type parasites (Daneshvar *et al*. 2003). Attenuation of virulence of *Theileria annulata* has been demonstrated following prolonged *in vitro* passage of schizont-infected cells (Hall *et al*. 1999). *Babesia bovis* and *Babesia bigemina* have both shown reduced virulence after repeated passage through splenectomized cattle (Pipano *et al*. 2002). Vaccination against *Toxoplasma gondii*-induced abortion in sheep has been achieved using a live incomplete (S48) strain of the parasite (O'Connell *et al*. 1988; Wilkins *et al*. 1988). The S48 strain of *T. gondii* was passaged over 3000 times in mice and has lost the ability to form both tissue cysts and oocysts (Buxton, 1993). A commercial vaccine based on this product is currently available in several countries worldwide and is licensed for veterinary use only.

The purpose of our study was to determine whether prolonged *in vitro* culture of *N. caninum* tachyzoites by repeatedly passaging parasites into fresh cell monolayers, would lead to attenuation of virulence *in vivo*. This study compared tachyzoites that were passaged successively between 33 and 39 times (low passage) and tachyzoites passaged between 70 and 84 times (high passage).

MATERIALS AND METHODS

Parasites and experimental inocula

Neospora caninum tachyzoites (NC1 isolate) (Dubey *et al*. 1988) were successively propagated as previously described (Innes *et al*. 1995). Briefly, *N. caninum* parasites were cultured in Vero cell monolayers in 25 cm^2 canted neck tissue-culture flasks (Corning, NY, USA). The monolayers were then disrupted using a sterile cell scraper (Corning, NY, USA), parasites were counted in a Neubauer haemocytometer and resuspended in phosphate-buffered saline (PBS) to produce inocula containing either 5×10^7 or 1×10^8 tachyzoites per ml. The control inoculum contained the same number of Vero cells as was present in the parasite inoculum and was prepared in the same manner. Both the parasites and Vero cells were inoculated into mice intraperitoneally (i.p.) within 1 h of their preparation in the laboratory, in a volume of 100 μl per mouse. During the routine passage of the parasites, aliquots were cryopreserved at regular intervals and stored in liquid nitrogen as described below.

The parasites described in this paper were isolated from a single source. The passage numbers refer to the continuous *in vitro* passage of the same stock of parasites for different lengths of time.

Cryopreservation and resuscitation of N. caninum *tachyzoites*

Infected monolayers were disrupted to release *N. caninum* tachyzoites as previously described (Innes *et al*. 2001). The tachyzoites were then counted and concentrated by centrifugation at 630 ***g*** for 5 min. The supernatant was discarded and the parasites resuspended at a concentration of 1×10^7/ml in freezing medium (45% Iscoves modified Dulbecco's medium (IMDM) (Gibco BRL, Paisley, UK) 100 U/ml penicillin and 100 μg/ml streptomycin, (Northumbria Biologicals, Cramlington, UK), 45% foetal bovine serum (FBS) (Labtech, Austria) and 10% dimethyl sulphoxide (DMSO) (Sigma, Irvine, UK)). The parasites were aliquotted into 1 ml cryotube vials (Nunc, Roskilde, Denmark) and placed in 1 °C cryofreezing containers (Nalgene, USA) stored at −70 °C for 24 h, then transferred to vapour-phase liquid nitrogen storage. The parasites were resuscitated from liquid nitrogen storage by being rapidly warmed to 37 °C, then washed in 10 ml of IMDM supplemented with 5% FBS. Following centrifugation as above, parasites were resuspended in 2 ml of IMDM supplemented with 5% FBS, then inoculated into two 25 cm^2 canted neck tissue culture flasks (Corning, NY, USA), each seeded 24 h previously with 1×10^5 Vero cells.

Experimental design

Female Balb/c mice, approximately 12 weeks old, were randomly assigned into groups of 10, identified by ear-marking, and fed rodent proprietary mix and fresh water *ad libitum*. The mice were inoculated intraperitoneally (i.p.) and observed daily. The morbidity of the animals was assessed according to a system agreed with the UK Home Office Inspectorate, (Table 1). A cumulative score of 5 on any day or a score of 4 for 2 consecutive days resulted in the mouse being culled. All surviving mice were euthanized on day 28 post-inoculation (p.i.) by CO_2 inhalation. At post-mortem, samples of brain were removed and stored at −20 °C for analysis by *N. caninum* polymerase chain reaction (PCR). Brain, lung, liver and kidney samples were also removed and stored in 10% formal saline for

Table 1. Morbidity score categories in accordance with guidelines agreed by the UK Home Office Inspectorate

Category	Description	Score
A	Sleek/glossy coat	0
Febrile response	Ruffled coat	1
	Stary stiff coat	2
B	Weight maintained at pre-infection level	0
Dehydration/ Inappetence	10% weight loss	1
	20% weight loss	2
C	Bright and active	0
Demeanour	Hunched	1
Scoring cumulative in this category	Tottering gait	1
	A reluctance to move	1

Total Score = A + B + C.

Table 2. Experimental design

Experiment	Group	*n*	Inoculum*
1	1	10	5×10^6 low-passage (LP) (p.33) tachyzoites
	2	10	5×10^6 high-passage (HP) (p.70) tachyzoites
	3	10	1×10^6 Vero cells
2	4	10	5×10^6 LP (p.39) tachyzoites
	5	10	5×10^6 HP (p.84) tachyzoites
	6	10	1×10^7 LP (p.39) tachyzoites
	7	10	1×10^7 HP (p.84) tachyzoites
	8	10	$4{\cdot}9 \times 10^5$ Vero cells

* (p.33) = Passage number 33; (p.70) = passage number 70; (p.39) = passage number 39 and (p.84) = passage number 84.

histopathological examination, and blood was drawn from the heart to obtain serum.

Groups of mice were inoculated i.p. with different doses of low-passage (LP) and high-passage (HP) parasites or a control inoculum of Vero cells as indicated in Table 2.

ITS1 PCR

DNA was extracted from 25 mg samples of brain using the DNeasy Kit (Qiagen) as per manufacturer's instructions. Following extraction the DNA was stored at −20 °C prior to analysis by PCR. A nested PCR was used to detect *N. caninum*-specific internal transcribed spacer 1 (ITS1) DNA (Holmdahl and Mattsson, 1996) using the method previously described by Buxton *et al.* (1998). This produced a band of 297 bp when the products (5 μl each) were analysed by agarose gel electrophoresis (1·8%), stained with ethidium bromide and visualized under UV light.

Samples for histology and immunohistochemistry

Post-fixation, the brains were sliced coronally and, along with blocks of the other tissues, were processed to paraffin wax. Sections 5 μm thick were cut and stained with haematoxylin and eosin (H&E). To detect *N. caninum*, specific immunohistochemistry was used as previously described (Buxton *et al.* 1997). Briefly, tissue sections were incubated at 4 °C overnight with a primary polyclonal rabbit antiserum to *N. caninum* (diluted 1 : 1000). The slides were washed in 0·5 M sodium chloride in 0·01 M phosphate buffer, a biotinylated goat-anti-rabbit IgG was used as a secondary antibody. Bound antibody was detected using the commercially available Vector-elite ABC system (Vector Laboratories, Peterborough, UK), with diaminobenzidine (DAB) being used as the chromogen. Sections were then counterstained with Mayer's haematoxylin and mounted under coverslips.

Serology

Blood taken at post-mortem was collected into sterile 1·5 ml tubes, allowed to clot overnight at 4 °C and then centrifuged at 5000 ***g*** for 2 min. The serum was decanted and stored at −20 °C prior to analysis. An indirect fluorescent antibody test (IFAT) for the detection of IgG was carried out as previously described (Buxton *et al.* 1997). Test sera were titrated in 2-fold dilutions from 1 : 16 to a final concentration of 1 : 4096. The endpoint was determined as the final concentration demonstrating distinct whole tachyzoite fluorescence (Conrad *et al.* 1993). The same procedure was used for the detection of IgM with the exception that fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM was used as the secondary antibody.

In vitro *growth characteristics*

The *in vitro* growth characteristics of the parasites were determined by quantifying their differential incorporation of tritiated [^{3}H]uracil. Three replicate experiments were undertaken for low passage parasites (p.37, p.38 and p.39) and for high passage parasites using (p.74, p.75 and p.76). The procedure was carried out as previously described (Innes *et al.* 1995). In brief, Vero cells were cultured at a concentration of 5×10^4 cells per well, in 96-well flat bottomed micro-titre plates (Nunc, Roskilde, Denmark) for 24 h prior to inoculation with the parasites. Parasites were added to quadruplicate wells containing Vero cells at a parasite to cell ratio of 4 tachyzoites: 1 cell. The cultures were labelled with 37 kBq of [^{3}H]uracil per well (Amersham, Bucks, UK) and incubated at 37 °C in a humidified

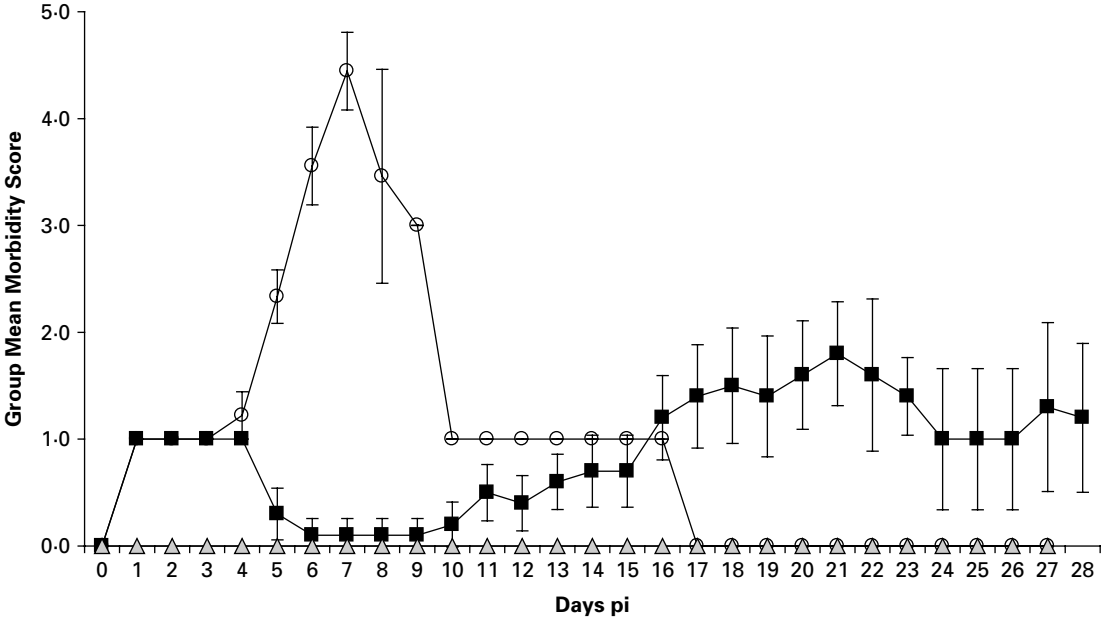

Fig. 1. Mean group morbidity score in Experiment 1. ○ – Group 1 (5×10^6 LP). ■ – Group 2 (5×10^6 HP). △ – Group 3 (1×10^6 Vero cells). Error bars (S.E.M.).

5% CO_2 atmosphere. Immediately prior to harvesting, parasite multiplication was stopped by the addition of 10 μl of 1 M sodium hydroxide. Cultures were harvested at 6, 12, 24, 48, 72 and 96 h post-inoculation onto glass-fibre filters and the parasite-associated radioactivity was quantified using a MATRIX 96^{TM} gas proportional counter (Canberra Packard, Meriden CT, USA).

Statistical analysis

The mortality data from both Experiments 1 and 2 were analysed using the Kaplan-Meier procedure. In Exp. 2, mice were generally weighed on alternate days. A repeated measures model was fitted to data for days 2, 4 and 6 p.i. only as no mice in Group 6 (1×10^7 LP) survived beyond day 7 p.i. The lack of independence between observations on the same mouse was modelled using an autoregressive type 1 correlation structure. To allow for the different mean starting weights for the groups, the day 0 reading was included in the model as a covariate. For ease of interpretation the results have been presented graphically as percentage changes from baseline. Analysis of variance (ANOVA) was used to analyse the *in vitro* growth experiment and IgG, IgM IFAT results. For the *in vitro* experiment a mixed model was used: counts per minute were analysed on a log scale, with low/high passage fitted as a fixed effect and the replicates within each type of passage fitted as random effects. A one-way ANOVA was used for the IgG and IgM data with the age of the animal at sampling being included in the model as a covariate. Differences in morbidity scores between two groups over successive times were assessed using Mann-Whitney tests. To account for multiple comparisons, the false discovery control method of Benjamini and Hochberg (1995) was used, as justified in this context by Benjamini and Yekutieli (2001). All statistical analyses were undertaken using Genstat 8th Edition apart from the false discovery rate procedure that was implemented within Microsoft Excel.

RESULTS

Exp. 1. Comparison of morbidity and mortality in mice inoculated with 5×10^6 low- or high-passage N. caninum *tachyzoites*

Clinical observations. Low-passage parasites. Group 1 mice (5×10^6 LP) developed ruffled coats from day 1 p.i., and from day 4 p.i. additional clinical symptoms were observed including hunching, reluctance to move, tottering gait and a stiff stary coat. A drop in group mean body weight of 6·6% (1·3 g) was observed by day 6 p.i. (data not shown). Animals were scored and culled in accordance with the scheme in Table 1 and results are illustrated in Fig. 1. By day 9 p.i. only 1 of the original 10 mice remained (Fig. 2). This animal became asymptomatic on day 17 p.i., and remained so until the end of the experimental period.

High-passage parasites. Group 2 mice (5×10^6 HP) had less severe clinical symptoms of infection, when compared to the group 1 animals. Ruffled coats were seen on days 1–4 p.i., with the animals becoming asymptomatic on day 5 p.i. From day 11 p.i., symptoms including ruffled coats, hunching,

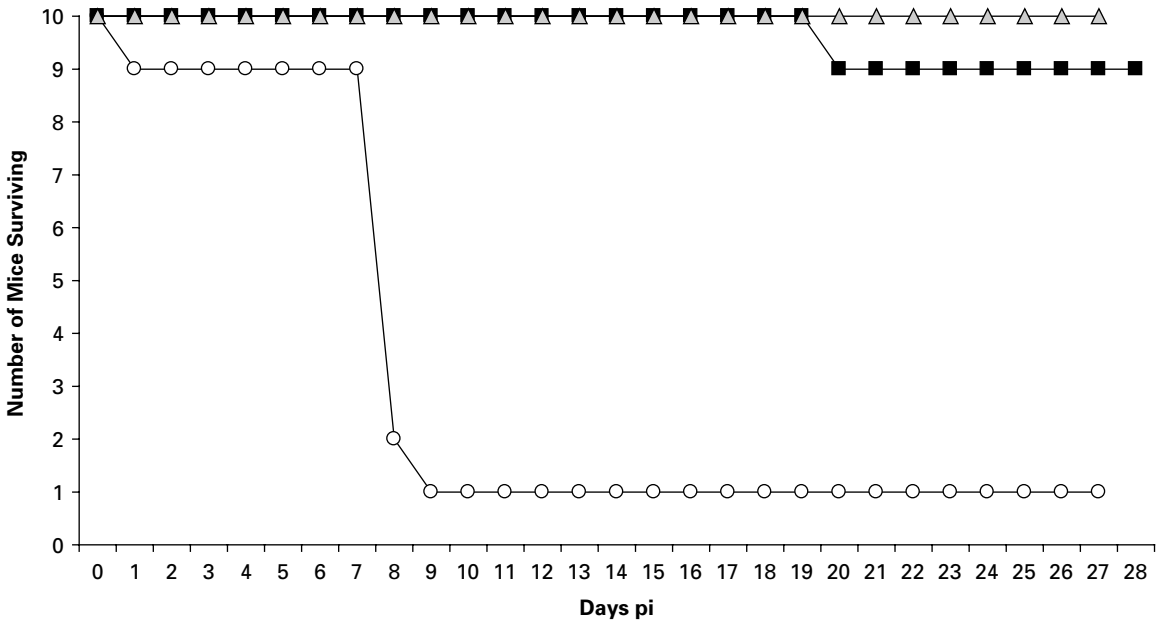

Fig. 2. Mortality rates in Experiment 1. ○ – Group 1 (5×10^6 LP). ■ – Group 2 (5×10^6 HP). △ – Group 3 (1×10^6 Vero cells).

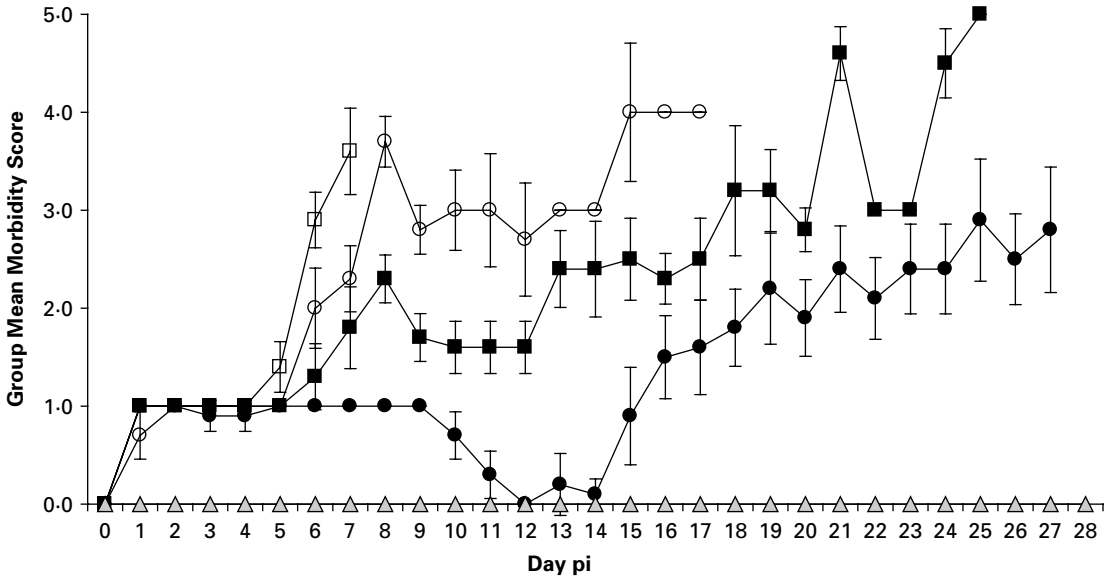

Fig. 3. Mean group morbidity score in Experiment 2. ○ – Group 4 (5×10^6 LP). ● – Group 5 (5×10^6 HP). □ – Group 6 (1×10^7 LP). ■ – Group 7 (1×10^7 HP). △ – Group 8 ($4{\cdot}9 \times 10^5$ Vero cells). Error bars (S.E.M.).

a reluctance to move, and a tottering gait were observed in a few mice and were responsible for a group mean score of between 1 and 2 being maintained to the end of the experiment (Fig. 1). Only 1 of the 10 mice in group 2 died, and this occurred on day 23 p.i. (Fig. 2). Group 2 had a maximum group mean weight loss of 2·8% (0·6 g) on day 21 p.i. and by day 28 p.i. had returned to the group mean starting weight (data not shown).

Control animals. The control animals (group 3) showed no clinical symptoms and maintained a stable body weight throughout the experiment.

Exp. 2. Comparison of morbidity and mortality in mice inoculated with different doses of low- or high-passage N. caninum *tachyzoites*

This experiment examined whether the difference in virulence demonstrated in Exp. 1 could be repeated following cryopreservation and resuscitation of parasites and using different doses of inoculum.

Low-passage parasites. Group 4 mice (5×10^6 LP) displayed symptoms from day 4 p.i., including hunching, reluctance to move, tottering gait and a stiff stary coat (Fig. 3). A decrease in group mean

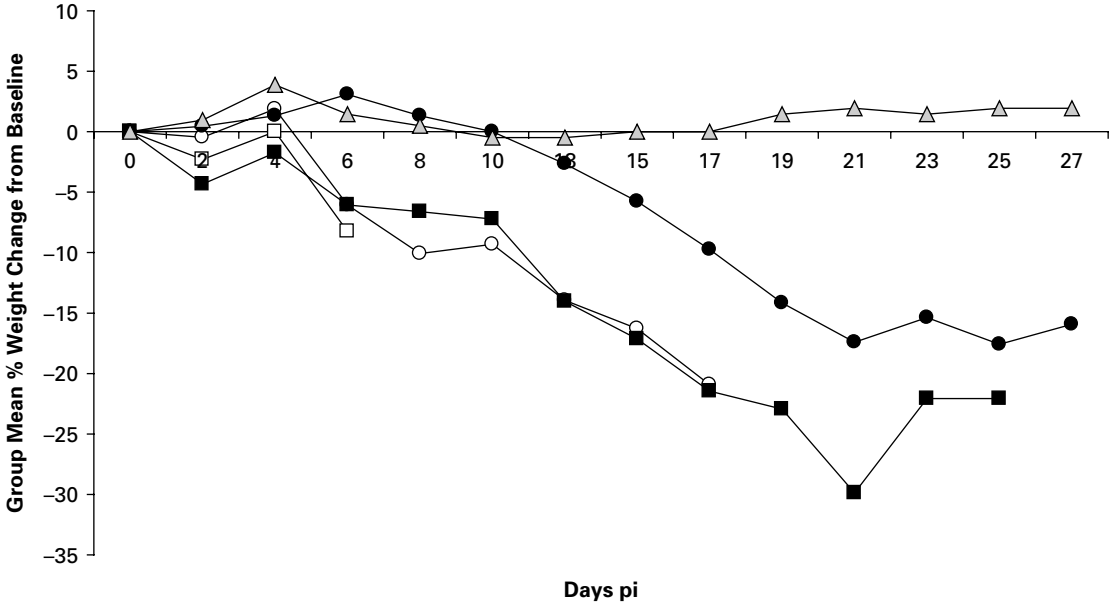

Fig. 4. Mean group % weight change from baseline in Experiment 2. ○ – Group 4 (5×10^6 LP). ● – Group 5 (5×10^6 HP). □ – Group 6 (1×10^7 LP). ■ – Group 7 (1×10^7 HP). △ – Group 8 ($4{\cdot}9 \times 10^5$ Vero cells).

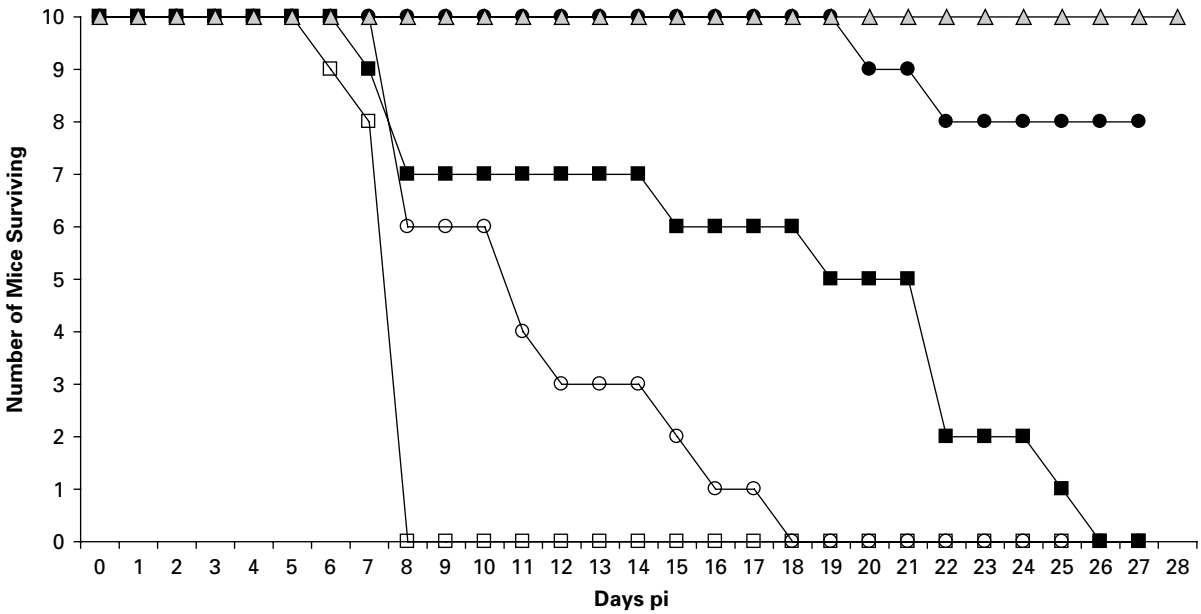

Fig. 5. Mortality rate in Experiment 2 ○ – Group 4 (5×10^6 LP). ● – Group 5 (5×10^6 HP). □ – Group 6 (1×10^7 LP). ■ – Group 7 (1×10^7 HP). △ – Group 8 ($4{\cdot}9 \times 10^5$ Vero cells).

body weight of 20·9% (4·5 g) (Fig. 4) was also observed. All 10 animals in group 4 were culled or died by day 17 p.i. (Fig. 5).

Group 6 mice (1×10^7 LP) had more severe clinical symptoms by day 5 p.i. (Fig. 3), including an 8·3% (1·8 g) drop in group mean body weight between days 4 and 6 p.i. (see Fig. 4). All 10 mice were culled or died by day 8 p.i. (Fig. 5).

High-passage parasites. Group 5 mice (5×10^6 HP) displayed milder symptoms of murine neosporosis than group 4, symptoms included a ruffled coat, weight loss and reluctance to move which were observed throughout the experiment (Fig. 3). The mean morbidity score for group 5 was significantly lower ($P < 0{\cdot}05$) (using Benjamini and Hochberg's false discovery control method) between 6 and 11 days p.i., compared to that seen in the animals in group 4 (5×10^6 LP). A drop in group mean body weight was observed from day 12 p.i., reaching a maximum mean decrease of 17·6% (3·9 g) on day 25 p.i. (see Fig. 4). Only 2 mice in group 5 were culled or died prior to day 28 p.i. (Fig. 5). When the survival times from groups 4 and 5 were compared

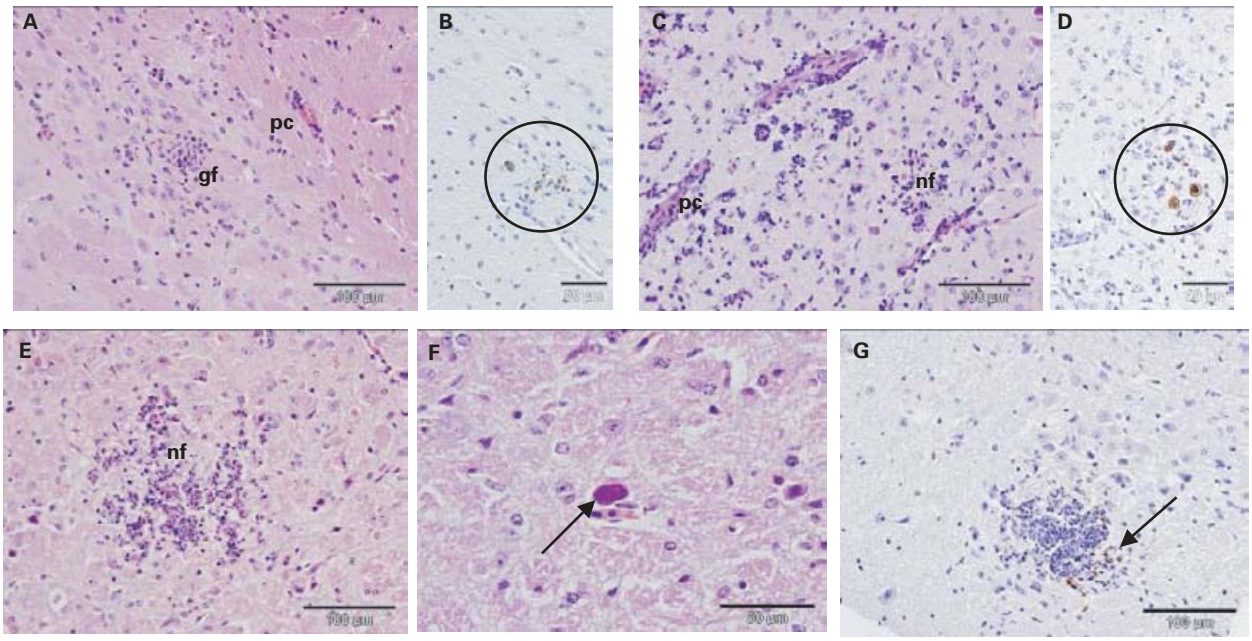

Fig. 6. Group 4 (5×10^6 LP) and Group 6 (1×10^7 LP). (A). Brain from a mouse culled at day 7 p.i. showing small glial focus (gf) and a mild perivascular cuff (pc), (H&E). (B). Tissue cyst and tachyzoites within a small focus of gliosis in the brain of the same mouse (indicated within circle) (IHC). (C) Brain from a mouse that was culled at day 18 p.i. showing severe foci of necrosis (nf) and perivascular cuffing by mononuclear cells (pc) (H&E). (D) Small clumps of *Neospora caninum* parasites within lesions in the brain of the same mouse (indicated within circle) (IHC). (E). Brain from a mouse culled at day 7 p.i. showing a focus of necrosis. (F). *N. caninum* tissue cyst (arrow) (H&E). (G). Small aggregates of *N. caninum* parasites (arrow) within a focus of necrosis in the brain of the same mouse (IHC).

using the Kaplan-Meier procedure, group 5 was shown to have survived significantly longer ($P<0{\cdot}001$) than group 4.

Group 7 animals (1×10^7 HP) showed fewer symptoms than either group 4 or group 6 (Fig. 3). The mean morbidity score for group 7 was significantly lower ($P<0{\cdot}05$) between 6 and 7 days p.i., compared to that seen in the animals in group 6 (1×10^7 LP) (using Benjamini and Hochberg's false discovery control method). This dose of the high-passage parasites, however, resulted in a 29·9% (6·9 g) drop in group mean body weight (Fig. 4), and all 10 mice in this group died or were culled by day 25 p.i. (Fig. 5). When the survival times from groups 6 and 7 were compared using the Kaplan-Meier procedure, group 7 was shown to have survived significantly longer ($P=0{\cdot}006$) than group 6.

Control animals. The control animals (group 8) displayed no clinical symptoms and maintained a stable body weight throughout the experiment.

Histopathology and PCR

Histological examination was performed on all samples collected and the extent of pathology, was based on the number and size of lesions observed in the brain. Lesions in the tissues were assessed histologically and the presence of parasites was demonstrated by immunohistochemistry. In all groups the severity of lesions was related to the time of survival. In general, mice culled before day 12 p.i. had mild or no lesions, whereas mice that survived for 15 days or longer had more severe pathology.

Low-passage parasites. The mice from group 4 (5×10^6 LP) that died before day 12 p.i. showed either mild, (Fig. 6A), or no pathology. The brains from the 2 mice that survived to days 15 and 18 p.i. had severe lesions, characterized by mononuclear perivascular cuffs and necrotic foci (Fig. 6C). Parasite tissue cysts or tachyzoites seen in H & E sections were confirmed by means of IHC (Fig. 6B and D).

Group 6 mice (1×10^7 LP) were all culled at day 7 p.i., 6 mice had mild lesions characterized by small to moderate numbers of foci of gliosis and necrosis (Fig 6E), whilst in 2 mice lesions and parasites were not detected. Parasite tissue cysts or tachyzoites were detected in 5 mice by histology (Fig. 6F) or IHC (Fig. 6G).

Parasite DNA was detected in 10 out of 10 brain samples from mice in group 4 (5×10^6 LP) and in 7 out of 8 brain samples in group 6 mice (1×10^7 LP).

High-passage parasites. Seven mice from group 5 (5×10^6 HP), culled at the end of the experiment (day 28 p.i.) were severely affected (Fig. 7A), characteristically with large mononuclear perivascular cuffs,

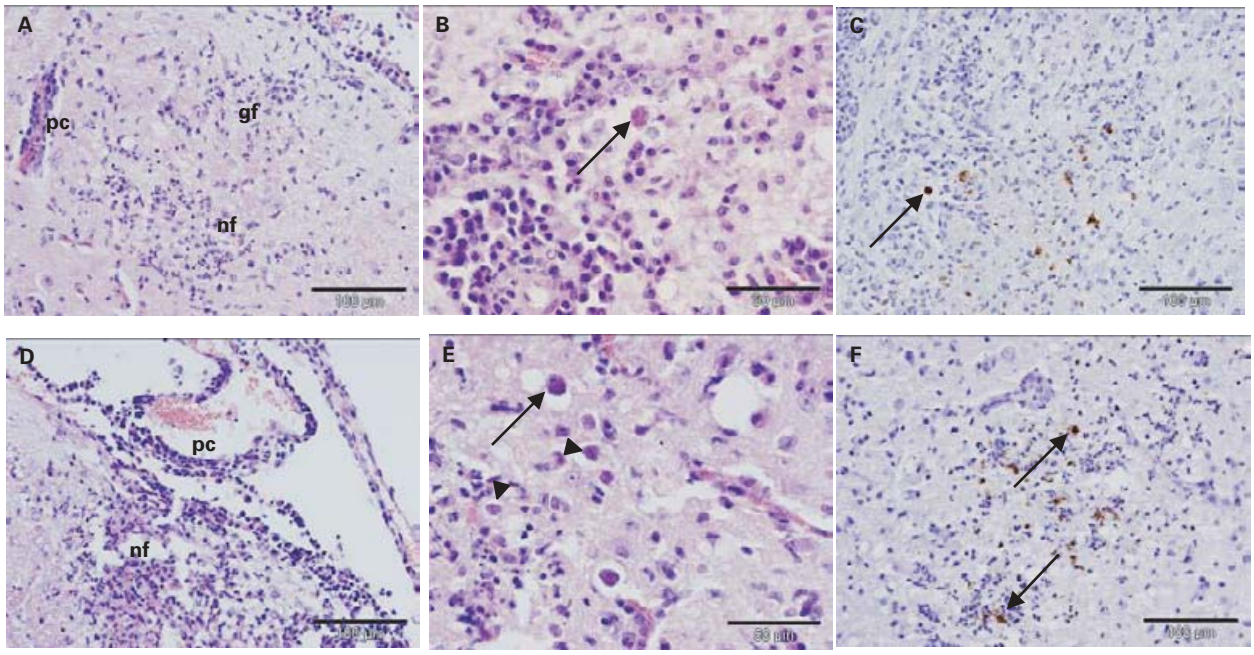

Fig. 7. Group 5 (5×10^6 HP) and Group 5 (1×10^7 HP). (A). Brain from a mouse culled at day 28 p.i. showing severe lesions characterized by focal gliosis and vascular inflammation. (B) A small tissue cyst (arrow) associated with a focus of inflammation in the brain of the same mouse (H&E). (C). A small tissue cyst (arrow) and numerous tachyzoites within an area of necrosis and inflammation in the brain of the same mouse (IHC). (D). Brain from a mouse culled at day 24 p.i. showing a severe focus of necrosis in the molecular layer of the cerebellum, with associated meningeal inflammation. (E). One tissue cyst (arrow) and tachyzoites (arrowheads) in parasitophorous vacuoles, in the brain of a mouse culled on day 24 p.i. (H&E). (F) *Neospora caninum* tissue cysts (arrows) and tachyzoites within parasitophorous vacuoles in a focus of necrosis in the brain of a mouse culled at day 21 p.i. (IHC).

necrotic and glial foci and mononuclear meningitis. Many tissue cysts and tachyzoites were also observed (Fig. 7B and C), while in 1 mouse in group 5 only mild histological changes were detected.

In group 7 (1×10^7 HP), 7 mice had similar lesions to those observed in group 5. These mice were culled between days 18 and 25 p.i. and frequent foci of severe necrosis with gliosis and perivascular cuffing were recorded (Fig. 7D). Many tissue cysts and tachyzoites were present, mainly associated with lesions (Fig. 7E and F). In the 2 mice culled at day 7 p.i. only small foci of mild gliosis were seen.

Parasite DNA was detected in 7 out of 8 brain samples in group 5 mice (5×10^6 HP) and in 9 out of 9 brain samples in group 7 animals (1×10^7 HP).

Control animals. The control animals in group 8 had no clinical pathology, lesions, detectable parasites or parasite DNA.

Serology

Low-passage parasites. Group 4 mice (5×10^6 LP) had IgM titres ranging from $<1:16$ to $1:128$ and IgG titres ranging from $<1:16$ to $1:64$. While group 6 (1×10^7 LP) had IgM titres ranging from $1:16$ to $1:256$ and IgG titres of between $<1:16$ and $1:16$. In general, low antibody titres were associated with early death or culling. The mouse in group 4 that survived to day 18 p.i. gave the highest IgG titre ($1:64$) whilst also giving to lowest IgM titre ($<1:16$).

High-passage parasites. Group 5 mice (5×10^6 HP) mice showed no detectable IgM (titre of $<1:16$) but had IgG titres ranging from $1:256$ to $1:1024$. Group 7 (1×10^7 HP), had IgM titres ranging from $<1:16$ to $1:256$ while the IgG titres in group 7 ranged from $1:256$ to $1:2048$.

When the IgG results were compared it was evident that group 5 produced greater levels of IgG than that produced by the animals in group 4, and group 7 produced greater levels of IgG than group 6. However, as IgG generally increased with time these differences are largely a reflection of the increased survival times in the groups receiving HP parasites.

Control animals. The control animals (group 8) had no detectable *Neospora*-specific IgM or IgG antibody titres.

In vitro *multiplication rates of parasites*

Parasite multiplication was measured by the differential incorporation of [^{3}H]uracil by the multiplying parasites, and expressed as counts per min. Both the low-passage (p.37–p.39) and the high-passage

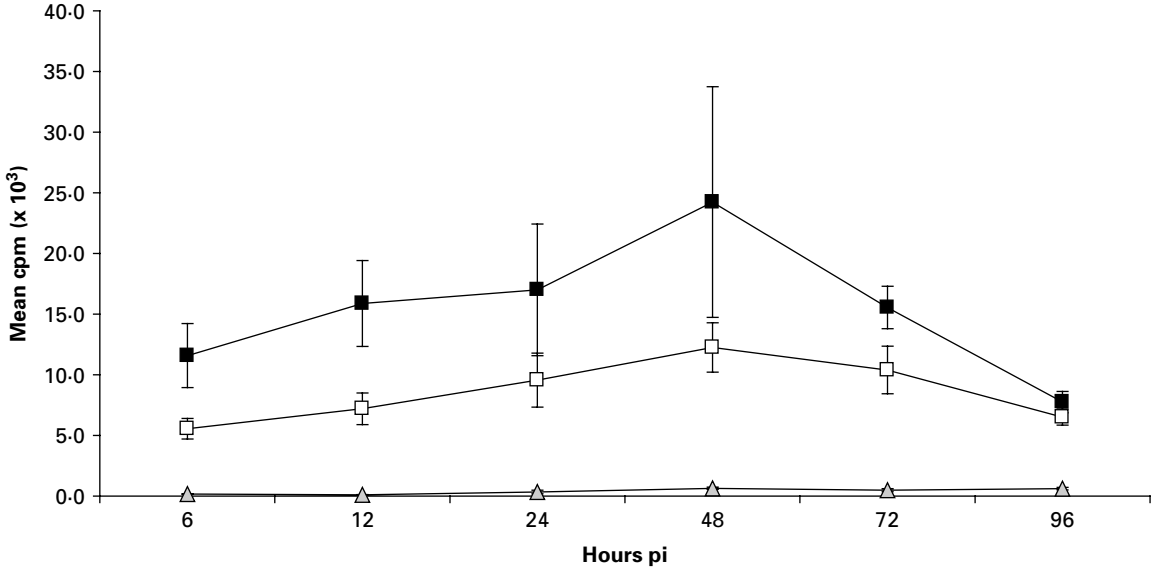

Fig. 8. *In vitro* growth rates of *Neospora caninum* tachyzoites assessed by the differential uptake of [^{3}H]uracil and expressed as counts per minute (cpm). □ – (low-passage tachyzoites p.37–p.39). ■ – (High-passage tachyzoites p.74–p.76). △ – (Vero cells) Error bars (S.E.M. from 3 separate experiments using quadruplicate samples).

(p.74–p.76) parasites multiplied steadily up to 48 h (Fig. 8), after which growth declined. There was strong evidence ($P<0{\cdot}001$) of a difference in the pattern of growth rates over time between the high- and low-passage parasites with the high-passage parasites multiplying faster than the low-passage parasites at every time-point. The decrease in multiplication seen towards the end of the experiments is likely to be due to the tachyzoites lysing the Vero cell monolayers. The uninfected Vero cell controls did not incorporate [^{3}H]uracil at any of the time-points tested.

DISCUSSION

Attenuation of virulence of *N. caninum* tachyzoites was achieved by continuous, passage of parasites in cell culture. In our study, differences were clearly demonstrated in the *in vivo* pathogenicity of *N. caninum* tachyzoites maintained for different lengths of time in tissue culture. The animals infected with the low-passage (virulent) parasites demonstrated a very rapid progression to severe clinical symptoms following inoculation with 5×10^6 parasites, resulting in 95·0% (19/20) of the animals (groups 1 and 4) dying or being culled within 28 days p.i. By contrast, animals inoculated with the high-passage (attenuated) parasites, showed milder clinical symptoms and a 15·0% mortality rate with only 3 of 20 animals in groups 2 and 5 dying or being culled before day 28 p.i. The attenuation effect appears to be dose dependent as the mice that received 1×10^7 high-passage parasites had all died by day 26 p.i. However, as a group they survived significantly longer than the mice that received 1×10^7 low-passage parasites which all died or were culled by day 8 p.i. The attenuation phenotype observed in our study appears stable following cryopreservation and resuscitation, as similar results were obtained in 2 separate experiments. Other studies have shown that a subcutaneous (s.c.) inoculation with 2×10^5 NC1 strain tachyzoites in Balb/c mice resulted in a mortality rate of 58% by day 70 p.i. (Lindsay *et al*. 1995). A subsequent paper reported a mortality rate of 70% in Balb/c mice within 41 days of an s.c. challenge with 5×10^5 NC1 strain tachyzoites (Lindsay *et al*. 1999). These papers show that an s.c. inoculation of as few as 2×10^5 NC1 strain parasites may be lethal in Balb/c mice, although no information is provided in these papers concerning the length of time the parasites had been maintained in culture prior to inoculation into the mice.

Symptoms of clinical neosporosis in mice include a rough coat, depressed appetite, dehydration, weight loss, tottering gait and reluctance to move (Lindsay and Dubey, 1989). Weight loss appears to be a characteristic symptom during a primary experimental infection with *N. caninum* tachyzoites in mice, as a 23% reduction in weight was observed by day 25 p.i. in Balb/c mice infected with NC Liverpool strain *N. caninum* parasites (Quinn *et al*. 2002). The initial weight loss that occurs during experimental *N. caninum* infections in mice is probably due to parasite multiplication causing tissue damage to the peritoneum and internal organs, leading to reduced appetite and dehydration. That this weight loss is due to tissue damage rather than the method of inoculation, is confirmed by the control animals, which received an i.p. inoculation of Vero cells but did not lose weight. Levels of weight loss may act as a useful quantifiable symptom of

disease, following a primary experimental challenge with *N. caninum* parasites.

Detection of specific antibodies in mice inoculated with *N. caninum* was related to survival time, with higher antibody titres recorded in mice inoculated with HP parasites. The humoral immune response was characterized by an initial rise in levels of IgM for the first few days following infection. The antibody response then switched to IgG at about 12 days p.i. Those mice that succumbed to infection early still demonstrated detectable IgM titres (ranging from 1 : 16 to 1 : 256) and had either undetectable or very low titres of IgG (ranging from $<1:16$ to 1 : 64). Higher specific antibody responses (both IgM and IgG) were seen in the mice inoculated with 1×10^7 HP *N. caninum* tachyzoites, compared to those inoculated with 5×10^6 HP *N. caninum* tachyzoites, suggesting that the antibody titre produced was related to the challenge dose of parasites. The importance of the humoral immune response during murine *Neospora* infections was demonstrated by Eperon *et al.* (1999), using B-cell deficient μMT mice, which showed increased susceptibility to infection with the parasite, compared to wild-type mice. The anti-parasite function of antibodies is yet to be determined, although a likely role may involve controlling the spread of extracellular parasites (Innes *et al.* 2005).

The severity of the pathology seen in our study appears to be related to the survival time in the mice, with those that survived longest demonstrating the most severe lesions. Similar histological observations to those described in our study were seen by Long *et al.* (1998), Lindsay *et al.* (1995, 1999) and Lundén *et al.* (2002) when examining CNS tissue from infected mice. In these studies mice that received a primary challenge with NC1 *N. caninum* tachyzoites developed severe neuropathological lesions, including focal inflammation, focal necrosis and perivascular cuffing. The findings from our study are in agreement with those of Long *et al.* (1998), who observed a positive correlation between the dose of parasite challenge and levels of neuropathology, with mice that received larger challenge doses exhibiting more severe pathology than mice that received lower challenge doses.

Positive *N. caninum* specific ITS1 PCR results were seen as early as day 7 p.i. in samples of brain from animals inoculated with either low-passage or high-passage parasites, demonstrating that both are capable of disseminating to the brains of infected animals. Parasite DNA was detected in the brains of 17/18 mice that received the low-passage (virulent) parasites (groups 4 and 6), and 16/17 mice that received the high-passage (attenuated) parasites. Tissue cysts containing *N. caninum* parasites were seen in the brains of mice infected with either the low-passage (virulent) parasites or the high-passage (attenuated) parasites, demonstrating that both are capable of stage differentiation. Loss of life-cycle stage differentiation has been seen with *T. gondii*, where in 1 case following as few as 35–40 passages of tachyzoites in mice, the parasite lost its ability to form oocysts when the tissue cysts were fed to cats (the definitive host of *T. gondii*) (Frenkel *et al.* 1976). However, these parasites still retained the ability to form tissue cysts in mice. The S48 strain of *T. gondii* (O'Connell *et al.* 1988; Wilkins *et al.* 1988) was passaged over 3000 times in mice (Bos, 1993) and was found to have lost the ability to differentiate into either oocysts or bradyzoites. As the S48 strain tachyzoites undergo limited multiplication *in vivo* but do not persist, they proved to be a very effective means of immunizing animals against *T. gondii* (Buxton and Innes 1995). This attenuated (S48) strain of *T. gondii* is marketed as Toxovax^{TM}, a successful tissue culture-grown vaccine capable of controlling *T. gondii* induced abortion in sheep (O'Connell *et al.* 1988; Wilkins *et al.* 1988; Bos, 1993; Buxton, 1993).

The comparison of *in vitro* growth rates demonstrated that the high-passage (attenuated) parasites multiplied faster than the low-passage (virulent) parasites throughout. This may be because the attenuated parasites had become more adapted to tissue culture. A study by Schock *et al.* (2001) demonstrated no link between length of time in tissue culture and the *in vitro* growth rates of 6 different *N. caninum* isolates; although this study did not compare the growth rates of different passage numbers of the same isolate. The findings described in our paper suggest that the length of time in culture may have an effect on the pathogenicity of *Neospora* parasites *in vivo*. Long *et al.* (1998) noted that prolonged *in vitro* cultivation of *N. caninum* tachyzoites led to a reduction in virulence *in vivo*. However, Atkinson *et al.* (1999) demonstrated no attenuation effect *in vivo* of NC Liverpool strain *N. caninum* following 14 months of continuous *in vitro* culture, when compared to a culture of NC Liverpool stored in liquid nitrogen during this time. The same authors showed that there were considerable differences in the *in vivo* pathogenicity of NC Liverpool strain *N. caninum* in mice compared to the NC-SweB1 strain (Atkinson *et al.* 1999).

The mechanisms involved in the attenuation of other protozoan parasites are still not fully understood. Daneshvar *et al.* (2003) demonstrated attenuation of *L. mexicana* and *L. major* through repeated *in vitro* culture of promastigotes in the presence of gentamicin. Attenuation of *T. annulata* parasites has also been achieved through prolonged *in vitro* cell culture where the loss of virulence *in vivo* is thought to be a complex and gradual process caused by changes in the interaction between parasite and host cells (Preston *et al.* 2001). The less severe lesions observed following inoculation of the attenuated strain of *T. annulata* into cattle has also

been linked with a reduced expression of matrix metalloproteases, which are required in cell adhesion and migration (Hall *et al*. 1999).

Lindsay *et al*. (1999) characterized 3 *N. caninum* temperature-sensitive mutants (NCts-4, NCts-8 and NCts-12) produced following exposure of parasites to *N*-methyl-*N*-nitro-*N*-nitrosoquanidine followed by 3–8 months continuous cell culture at 32·5 °C. These temperature-sensitive mutant parasites caused less severe lesions in Balb/c mice when compared to wild type NC1 strain parasites. However NCts-4, and NCts-12 reverted to the virulent wild type phenotype following culture at 37 °C, while NCts-8 showed a significantly reduced incidence of reversion to virulence following culture at 37 °C. This study by Lindsay *et al*. (1999) also demonstrated that vaccination of Balb/c mice with the NCts-8 parasites induced significant protection against a challenge with wild type NC1 strain *N. caninum* parasites. Live attenuated strains of intracellular protozoan parasites are attractive vaccine candidates as they are more likely to induce protective cell-mediated immune responses compared to immunization with killed vaccine preparations.

An interesting question arising from this study is whether the attenuation phenotype is due to the selection of a single genetic clone from a heterogeneous mix of parasites, or if the starting material was already clonal, then attenuation must have occurred through a more complex process such as loss or mutation of genes.

The results from our study show that it is possible to attenuate the virulence of *N. caninum* tachyzoites for mice through prolonged *in vitro* cell culture. The stability of the phenotype and the mechanisms involved in the attenuation process are still unknown and are the subject of continuing study.

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3 Inoculation of Balb/c mice with live attenuated tachyzoites protects against a lethal challenge of *Neospora caninum*

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2007

Parasitology

3.1 Manuscripts main hypotheses

The main hypotheses that were tested in this manuscript are

1. Can inoculation of mice with a low dose ($1x10^6$) of live virulent or attenuated *Neospora caninum* tachyzoites protect against a subsequent known lethal challenge with the parasite?
2. Does prior exposure to a low dose of virulent or attenuated *Neospora caninum* tachyzoites result in a reduction in the severity of histopathological lesions in the brains of animals following a lethal challenge with the parasite?
3. Does prior exposure to a low dose of virulent or attenuated *Neospora caninum* tachyzoites result in a reduction in the incidence lesions in the brains of animals which contain parasites (tachyzoites) following a lethal challenge with the parasite?
4. Does prior exposure to a low dose of virulent or attenuated *Neospora caninum* tachyzoites result in a reduction in the formation of tissue cysts in the brains of animals following a lethal challenge with the parasite?

This study clearly shows that prior exposure to a low dose ($1x10^6$) of either live virulent or attenuated tachyzoites conferred complete protection against death, as both vaccinated groups suffered 0% mortality following the lethal challenge. However animals from both groups still demonstrated severe histopathological lesions, and the presence of parasites / parasite antigens (as demonstrated using IHC). Though a

vaccination with attenuated parasites was unable to completely ameliorate the effects of the lethal challenge, animals receiving the attenuated vaccination generally demonstrated milder pathology and had a lower incidence of *Neospora* DNA positive brain samples compared to the animals vaccinated and challenged with the virulent parasites alone.

One of the major flaws with the experimental design of this experiment was: As NC1 strain parasites were used for both vaccination and challenge there was no way to determine if the parasites (parasite DNA) found in the brains of the animals were a result of the vaccination or the challenge. Had a different virulent *Neospora* strain been used for the challenge (i.e. Nc Liverpool), then RFLP or microsatellite technologies could have been used to differentiate the DNA from the two strains. This would have allowed a clearer measurement of the level of attenuation and whether the attenuated parasites still cause chronic infections.

3.2 Author contributions

PMB, SW, DB and EAI were involved in all aspects of the experimental design and planning of the experiments. All experiments were approved by Moredun Research Institutes experimental ethics committee.

PMB and SW inoculated all the mice and were responsible for making clinical observations, daily morbidity scoring, weighing of individual mice and the culling of animals when needed. At post mortem examination PMB and SW collected all tissue samples for PCR, qPCR and histological analysis and blood samples for serological screening.

PMB maintained the Vero cells and *Neospora* tachyzoites in tissue culture, enumerated the cells and tachyzoites using a Neubauer haemocytometer and prepared all of the inocula (Vero cells and tachyzoites) used during the experiments, extracted the DNA from all of the tissue samples examined and performed all ITS1 PCR and NC5 qPCR analysis. PMB collated all of the data and performed all of the statistical analysis (one-way ANOVA) on the morbidity, mouse weight change, and qPCR data and prepared all of the graphs and tables presented in the manuscript.

SW separated serum from clotted blood samples and performed all serological testing (IgM and IgG) by IFAT, while FC and DB performed all histological and immunohistological analysis and provided the images presented in the manuscript.

PMB, SW and EAI drafted the original manuscript with contributions from all other authors.

Inoculation of Balb/c mice with live attenuated tachyzoites protects against a lethal challenge of *Neospora caninum*

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SUMMARY

Neospora caninum tachyzoites attenuated through passage in tissue culture were tested for their ability to induce protective immunity against a lethal challenge dose of parasites. Balb/c mice were each inoculated with either 1×10^6 live virulent tachyzoites (Group 1) or 1×10^6 live attenuated tachyzoites (Group 2), while (Group 3) received a control inoculum. All mice were each challenged 28 days later with 5×10^6 virulent parasites. Histopathological lesions in the brains including necrosis and microgliosis were observed following post-mortem on day 28 post-challenge (p.c.) in 71% of Group 1 and 56% of Group 2. Immunohistochemistry (IHC) of these lesions showed tachyzoites and *Neospora* antigens to be associated with moderate brain lesions in 17% of Group 1, while in 11% of Group 2 *N. caninum* tissue cysts were detected, but these were not associated with lesions, Parasite DNA was detected by PCR in the brains of 86% of mice in Group 1 and 56% of mice in Group 2. Following challenge the mice in Group 3 showed high morbidity and 100% mortality within 17 days p.c. Positive IHC for *N. caninum* was seen in 88% of the Group 3 mice and parasite DNA was detected in all brain samples. This study shows that it is possible to protect against a lethal challenge of *N. caninum* through inoculation with attenuated or virulent tachyzoites. However, more severe pathology developed in mice initially inoculated with virulent parasites following a secondary challenge, compared to mice initially inoculated with attenuated parasites.

Key words: *Neospora caninum*, attenuated tachyzoites, protective immunity.

INTRODUCTION

Neospora caninum is an obligate intracellular protozoan parasite, closely related to *Toxoplasma gondii*. First described in 1984 (Bjerkås *et al*.), it has a worldwide distribution and is a major cause of bovine abortion (Dubey *et al*. 2006). Infection can be transmitted either transplacentally from mother to foetus, or through ingesting feed or water contaminated with oocysts, shed by infected dogs (a definitive host of *N. caninum*) (McAllister *et al*. 1998).

Experiments with cattle have demonstrated that protective immunity can be induced to prevent the transplacental transmission of *N. caninum* following a challenge with the parasite (Innes *et al*. 2001; Williams *et al*. 2007). Williams *et al*. (2003) showed that while naturally infected cows challenged with *N. caninum* early in gestation (10 weeks) were protected against abortion, vertical transmission of the parasites to the foetus still occurred, These studies taken together show that cattle develop a degree of protective immunity following prior exposure to the parasite and lend weight to the hypothesis that vaccination is a feasible option for controlling the infection.

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Experimental mouse models of neosporosis offer an economic and convenient system for testing potential vaccination candidates. Live attenuated temperature-sensitive mutants (Lindsay *et al*. 1999), sublethal doses of live *N. caninum* tachyzoites (Lundén *et al*. 2002) and live γ-irradiated *N. caninum* tachyzoites (Ramamoorthy *et al*. 2006) have all induced protection in Balb/c mice against a lethal challenge with the parasite. Killed and subunit vaccines have also shown some success in inducing protective immune responses in mice. Studies using *E. coli* expressed recombinant antigens NCMIC3 (Cannas *et al*. 2003) and NCSRS2 (Pinitkiatisakul *et al*. 2005) and virus-vectored expression of recombinant NcGRA7 (Liddell *et al*. 2003; Jenkins *et al*. 2004) have all induced protection in mice against a challenge with *N. caninum*. However, Baszler *et al*. (2000) demonstrated an exacerbation of neurological disease in mice immunized with soluble *N. caninum* tachyzoite antigens and subsequently challenged with the parasite.

Attenuation of virulence through *in vitro* culture has been examined in a number of protozoan parasite species including *Theileria* (Preston *et al*. 2001) and *Leishmania* (Daneshvar *et al*. 2003). The main objectives of this study were to determine whether *N. caninum* parasites attenuated through prolonged *in vitro* culture (Bartley *et al*. 2006) will induce protection against a known virulent challenge and to

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Table 1. Experimental design

(NC1 p.39 tachyzoites) – passage number 39, (NC1 p.43 tachyzoites) – passage number 43, (NC1 p.88 tachyzoites) – passage number 88.)

		Inoculation	
Group	*n*	Primary	Challenge
1	10	1×10^6 NC1 p.43 (Virulent)	5×10^6 NC1 p.39 (Virulent)
2	10	1×10^6 NC1 p.88 (Attenuated)	5×10^6 NC1 p.39 (Virulent)
3	10	$2{\cdot}45 \times 10^4$ Vero cells	5×10^6 NC1 p.39 (Virulent)

compare any differences in the protective effects between the virulent and attenuated parasites.

MATERIALS AND METHODS

Parasites and experimental inocula

Neospora caninum tachyzoites (NC1 isolate) (Dubey *et al*. 1988) were maintained (Innes *et al*. 1995) and used to prepare experimental inocula (Bartley *et al*. 2006) as previously described. Briefly, *N. caninum* parasites cultured in Vero cell monolayers were disrupted using a sterile cell scraper (Corning, NY, USA), parasites were counted in a Neubauer haemocytometer and resuspended in phosphate-buffered saline (PBS) to produce inocula containing either 1×10^7 or 5×10^7 tachyzoites per ml. The virulent parasites were passaged 43 times in tissue culture (passage (p.43)) while the attenuated parasites were passaged 88 times (passage (p.88)). Prior to their use as experimental inocula, the virulent challenge parasites had been subcultured 39 times (passage (p.39)). In a previous study (Bartley *et al*. 2006), prolonged culture of *Neospora in vitro* resulted in attenuation of their virulence *in vivo*. The control inoculum contained the equivalent number of Vero cells present in the parasite preparations and was prepared in the same manner. Both the parasite and the Vero cells were inoculated into mice intraperitoneally (i.p.) within 1 h of their preparation in the laboratory, in a volume of 100 μl per mouse.

Experimental design

Female Balb/c mice, approximately 12 weeks old, were randomly assigned into groups ($n = 10$), individually identified by ear-marking and fed rodent proprietary mix and fresh water *ad libitum*. The mice were inoculated i.p. with live *N. caninum* tachyzoites (see Table 1) and observed daily for 28 days post-inoculation (p.i.) On day 28 all mice were challenged i.p. with live *N. caninum* tachyzoites (see Table 1), defined as day 0 post-challenge (p.c.) The morbidity of the animals was assessed according to a system agreed with the UK Home Office Inspectorate (Bartley *et al*. 2006). All surviving mice were euthanased on day 28 p.c. by CO_2 inhalation. At post-mortem examination, samples of brain were removed and stored at $-20\,^{\circ}$C for analysis by *N. caninum* internal transcribed spacer 1 (ITS1) and quantitative polymerase chain reaction (PCR). Samples of brain, lung, liver, kidney and spleen were also removed and stored in 10% formal saline for histopathological examination, and blood was drawn from the heart and the serum was separated and stored at $-20\,^{\circ}$C prior to testing.

Serology

Serum collected at the time of post-mortem examination as previously described (Bartley *et al*. 2006) was examined with an indirect fluorescent antibody test (IFAT) for the detection of *Neospora*-specific IgG and IgM (Buxton *et al*. 1997). Briefly, 5 μl volumes of a 1×10^7/ml suspension of 0·2% (v/v) formalin-treated *N. caninum* tachyzoites were applied to each well of a 15-well multitest slide (ICN Biomedicals, Aurora OH) and allowed to dry overnight. Test sera were titrated in 2-fold dilutions from 1 : 16 to a final concentration of 1 : 4096 before addition to wells and incubated for 30 min at room temperature. Slides were washed 3 times in PBS before being incubated for 30 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (diluted 1 : 50 in PBS). They were then washed 3 times in PBS before cover-slips were mounted with buffered glycerol and viewed under an Olympus BX50 fluorescence microscope, with a U-MNB filter cube, using a ×40 objective. The endpoint was determined as the final concentration of serum demonstrating distinct whole tachyzoite fluorescence (Conrad *et al*. 1993). The same procedure was used for the detection of *Neospora*-specific IgM with the exception that FITC-conjugated goat anti-mouse IgM was used as the secondary antibody.

Histology and immunohistochemistry

All samples were fixed and sectioned for histological and immunohistochemical analysis as described (Bartley *et al*. 2006).

ITS1 PCR

DNA was extracted from brain samples and stored at −20 °C prior to analysis by PCR (Bartley *et al*. 2006). A *Neospora*-specific nested-PCR was used to detect the internal transcribed spacer 1 (ITS1) gene (Holmdahl and Mattsson, 1996) using the method previously described by Buxton *et al*. (1998). This produced a band of 297 bp when the products were analysed by 1·8% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light.

Quantitative SYBR green PCR

A quantitative PCR (qPCR) for the NC5 sequence of *N. caninum* (Kaufmann *et al*. 1996) was performed using the method and oligonucleotide primers described by Collantes-Fernández *et al*. (2002). The 28S rRNA gene was chosen for the quantification of host DNA (Collantes-Fernández *et al*. 2002), while the primers to the 28S rRNA gene were also used as 'housekeeping genes', to act as a control to determine the presence of potential PCR inhibiting compounds in the extracted DNA samples.

To prepare standards, DNA was extracted from tissue-culture derived *Neospora* tachyzoites using the DNeasy Kit (Qiagen) as per manufacturers' instructions. Total DNA was determined by spectrophotometry (Beckman Coulter DU530), adjusted with distilled water to a concentration of 2000 pg/μl and used to make a serial logarithmic dilution (2000, 200, 20, 2, 0·2 and 0·02 pg/μl). Host genomic DNA was extracted and quantified as previously described from brain tissue of an uninfected mouse and used to prepare standards at 20, 10, 5, 2, 1 and 0·5 ng/μl.

The *Neospora* NC5 and 28s rRNA primers (MWG Biotech AG, Germany) were performed as separate assays, with each reaction containing 1× platinum SYBR green qPCR supermix UDG (Invitrogen) consisting of 0·75U platinum *Taq* DNA polymerase, 10 mM Tris-HCl, 25 mM KCl, 1·5 mM $MgCl_2$, 100 μM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP) and 200 μM dUTP, 0·5U uracil-DNA-glycosylase, 10 pmol of each forward and reverse primer, 6 μl ultra-pure distilled water and 5 μl DNA (25 μl total volume). All standards and samples were undertaken in duplicate. Amplifications were performed with an ABI 7000 prism sequence detector (Applied Biosystems), using the manufacturer's recommended protocol (2 min at 50 °C, 10 min at 95 °C, 40 cycles of 95 °C for 15 sec and 60 °C for 1 min), the calculated cycle threshold (Ct) was exported for analysis in Microsoft excel. Quantities of parasite and genomic DNA in samples were calculated by interpolation on the two standard curves, where Ct values were plotted against the log of known concentrations of *Neospora* and host genomic DNA respectively. Following amplification of both *Neospora* and host DNA, melting curves for each were generated by a stepwise increase in temperature from 55 °C to 95 °C, to ensure amplification resulted in a single PCR product and no primer-dimers.

Statistical analysis

One-way analysis of variance (ANOVA) was applied to the morbidity, weight change and on qPCR data using Minitab statistical software (v13.1).

RESULTS

Clinical observations

Group 1. The animals in group 1 showed no clinical symptoms following the primary inoculation (1×10^6 NC1 p.43 tachyzoites).

Following challenge (5×10^6 NC1 p.39 tachyzoites), clinical symptoms, including a ruffled coat and slight weight loss, were seen from day 1 post-challenge (p.c.), resulting in a group mean maximum morbidity score of 1·0 on day 1 (see Fig. 1), the animals in this group returned to being clinically normal on day 5 p.c. Changes in the mean group weight were calculated by comparing the weight at each time-point to the weight on day 0 p.c. Group 1 demonstrated a mean weight loss on day 2 p.c. of 0·3 g, which was regained by day 4 p.c., the mice ended the experiment 2·3 g heavier than on day 0 p.c. (see Fig. 2). Group 1 suffered 0% mortality following challenge, with all the mice ($n=7$) surviving to the end of the experiment (see Fig. 3).

Group 2. The animals in Group 2 showed no clinical symptoms following the primary inoculation (1×10^6 NC1 p.88 tachyzoites). Following challenge (5×10^6 NC1 p.39 tachyzoites), clinical symptoms including a ruffled coat and slight weight loss were seen from day 1 p.c., resulting in a group mean morbidity score of between 0·2 and 1·0, the mice returned to being clinically normal by day 18 p.c. (see Fig. 1), there was no significant difference in the morbidity scores of Groups 1 and 2 at any time-point tested following challenge. The weight loss seen in Group 2 reached a maximum of 1·4 g on day 2 p.c., this weight was regained throughout the course of the experiment and the mice ended the experiment 0·7 g heavier than on day 0 p.c. (see Fig. 2). There were no statistical differences in the mean group weights when groups 1 and 2 were compared. Group 2 suffered 0% mortality following challenge, with all the mice ($n=9$) surviving to the end of the experiment (see Fig. 3).

Group 3. The animals in Group 3 showed no clinical symptoms following the primary inoculation ($2{\cdot}45 \times 10^4$ Vero cells). Following challenge (5×10^6

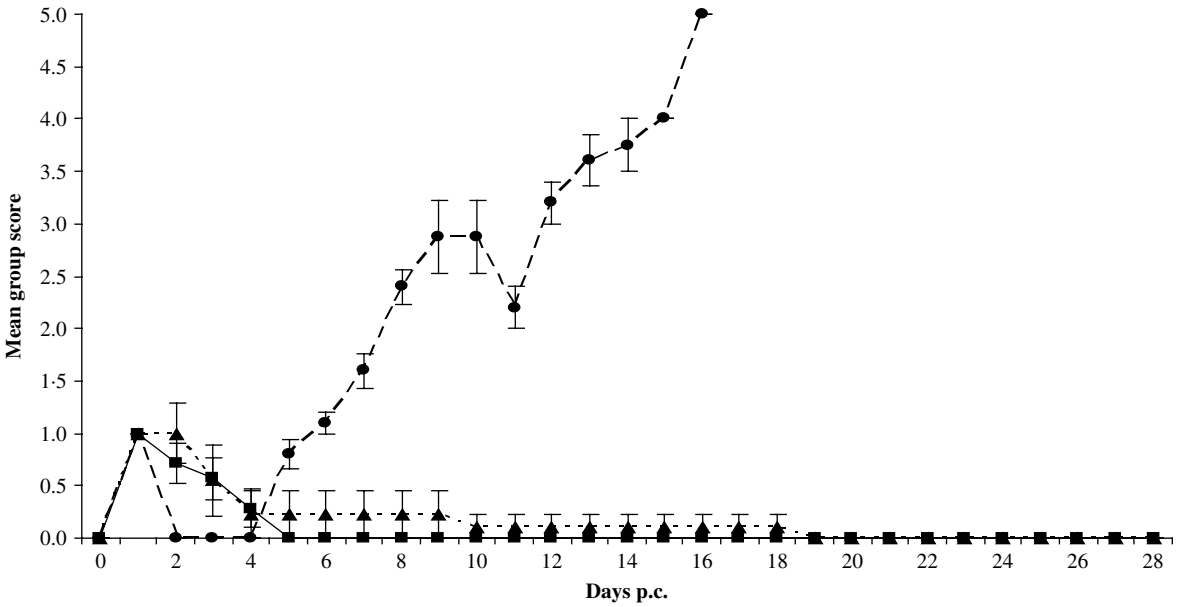

Fig. 1. Mean group morbidity scores following challenge with virulent *Neospora caninum* tachyzoites. ■ – Group 1, 1×10^6 NC1 p.43 tachyzoites. ▲ – Group 2, 1×10^6 NC1 p.88 tachyzoites. ● – Group 3, $2{\cdot}45 \times 10^4$ Vero cells. All groups were challenged with 5×10^6 NC1 p.39 tachyzoites. (Error bars ± S.E.M.).

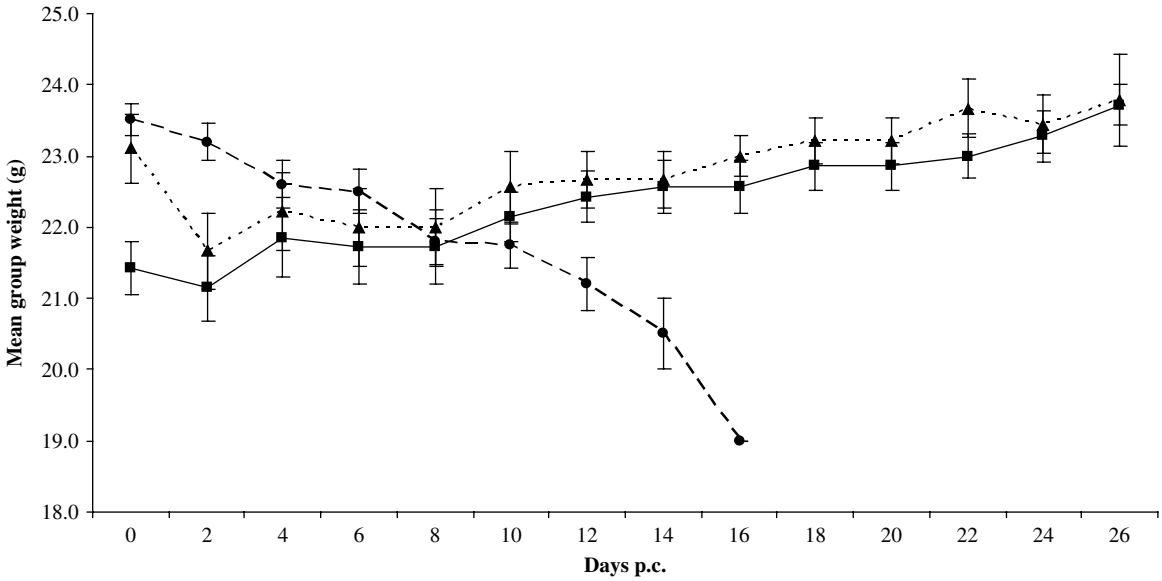

Fig. 2. Changes in mean group weight following challenge with virulent *Neospora caninum* tachyzoites. ■ – Group 1, 1×10^6 NC1 p.43 tachyzoites. ▲ – Group 2, 1×10^6 NC1 p.88 tachyzoites. ● – Group 3, $2{\cdot}45 \times 10^4$ Vero cells. All groups were challenged with 5×10^6 NC1 p.39 tachyzoites. (Error bars ± S.E.M.).

NC1 p.39 tachyzoites), clinical symptoms including a stiff stary coat were seen from as early as day 1 p.c. progressing through hunching, a tottering gait, a reluctance to move and weight loss; resulting in a group mean maximum morbidity score of 5·0 on day 16 p.c. (see Fig. 1). The weight loss started on day 2 p.c. with a mean group loss of 4·5 g by day 16 p.c. (see Fig. 2). The weight loss seen in Group 3 was significant ($P = 0{\cdot}001$) from day 12 p.c. onwards, when compared to Groups 1 and 2. Group 3 suffered 100% mortality following the parasite challenge, the first animals were culled on day 8 p.c. and the final mouse was culled on day 17 p.c. (see Fig. 3).

Three animals in Group 1 and 1 animal in Group 2 died on day 0 p.c. due to complications resulting from the administration of the challenge inoculum. Results from these animals were not used in any calculations.

Serology

IgM. *Neospora*-specific IgM titres of <1/16 were detected in all the mice from Group 1 ($n = 7$) and Group 2 ($n = 9$), while Group 3 had IgM titres of <1/16 ($n = 3$), 1/16 ($n = 2$) and 1/32 ($n = 3$).

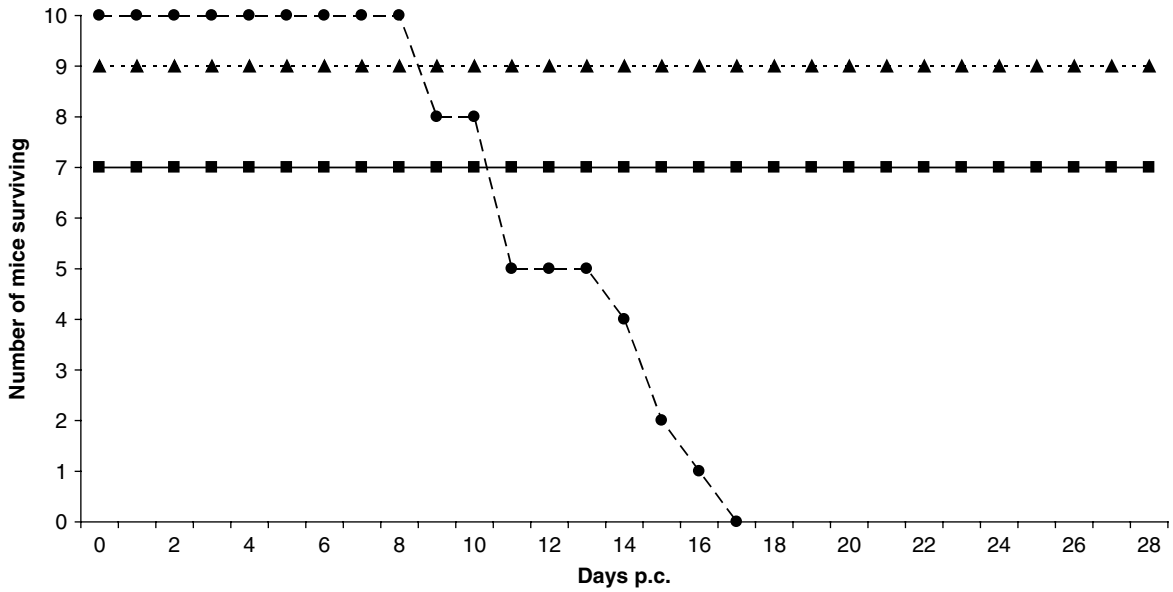

Fig. 3. Mortality rates following challenge with virulent *Neospora caninum* tachyzoites. ■ – Group 1, 1×10^6 NC1 p.43 tachyzoites. ▲ – Group 2, 1×10^6 NC1 p.88 tachyzoites. ● – Group 3, $2{\cdot}45 \times 10^4$ Vero cells. All groups were challenged with 5×10^6 NC1 p.39 tachyzoites.

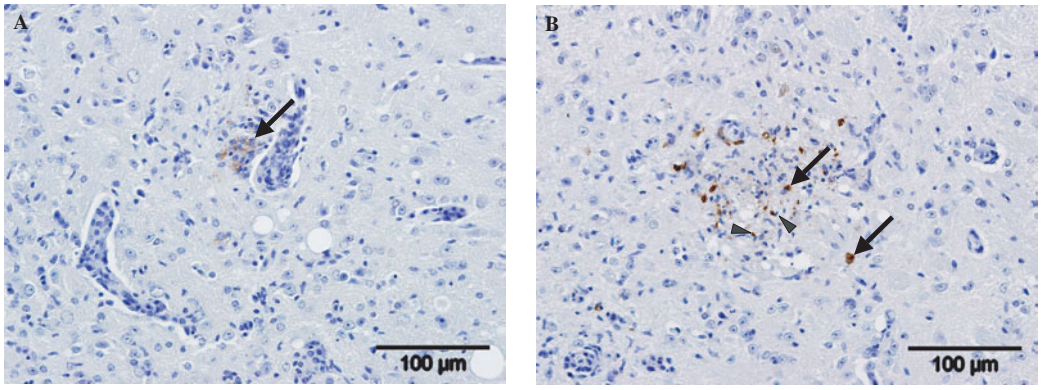

Fig. 4. (A) (Group 1). *Neospora* antigens (arrow) associated with mononuclear cells within a moderate brain lesion. (B) (Group 3). *Neospora* antigen associated with mononuclear cells; a tissue cyst (arrow) and tachyzoites (arrowhead) within a severe lesion.

IgG. Group 1 demonstrated *Neospora*-specific IgG titres that ranged between 1/1024 ($n=2$), 1/2048 ($n=3$) and 1/4096 ($n=2$), while Group 2 gave IgG titres of 1/1024 ($n=1$), 1/2048 ($n=5$) and 1/4096 ($n=3$). The IgG titres seen in the mice from Group 3 ranged from $<1/16$ ($n=3$) to 1/16 ($n=2$) and 1/32 ($n=3$). (We were unable to collect samples from 2 mice in Group 3.)

Pathology and PCR results

The extent of the histopathological changes post-challenge, based on the number and size of lesions observed in the brain, were assessed and the presence of the parasite was demonstrated by immunohistochemistry (IHC) and *Neospora*-specific PCR.

Group 1. Two animals in Group 1 had no demonstrable pathology, while a third mouse showed mild mononuclear perivascular cuffs. The remaining 4 mice in Group 1 showed large severe foci of mononuclear inflammation including perivascular cuffing; severe foci of necrosis and microgliosis. One of the mice with severe lesions also had foci of mineralization in the brain, while tachyzoites and parasite antigens were demonstrated in moderate lesions associated with mononuclear infiltrates in the brain of another severely affected mouse (see Fig. 4A). Positive *Neospora*-specific PCR results were obtained from 6 mice in this group (see Table 2), including 1 mouse in which lesions were not detected. One mouse was negative by PCR and by histopathology and IHC.

Group 2. Histopathological lesions were not detected in 4 of the mice from Group 2. In 1 mouse there was mild microgliosis along with mild mononuclear perivascular cuffs, while in another mouse there was moderate perivascular cuffing and

Table 2. *Neospora*-specific ITS1 PCR

		Neospora-specific ITS1 PCR results	
Group	*n*	Number positive	% Positive
1	7	6	86
2	9	5	56
3	8	8	100

microgliosis. In the 3 remaining animals in the group there were large severe foci of microgliosis, necrosis and mononuclear perivascular cuffs. Parasite antigens were detected in the brains of these 3 mice by IHC, but only in a very small number of cells and these were not associated with any lesions. In 1 of the mice with severe lesions a small tissue cyst was observed and was associated with inflammation but no necrosis or microgliosis. Positive PCR results were obtained from 5 mice in this group (see Table 2) and all 5 demonstrated pathological changes. Four mice gave negative PCR results; these animals also had no pathological changes.

Group 3. One mouse in Group 3 (post-mortemed on day 10 p.c.) showed no pathology in the brain, but had moderate numbers of *N. caninum* parasites in the lungs. Two other mice also culled on day 10 p.c. showed foci of mild mononuclear perivascular cuffs, mild microgliosis, and mild necrosis. The remaining 5 mice in the group were all culled between days 13 and 17 p.c. and demonstrated moderate to severe mononuclear perivascular cuffs, microgliosis, and necrosis. A number of these mice also showed signs of splenic haemorrhaging and hepatic vacuolation. Parasite cysts were detected in 5 animals, 2 of these animals were post-mortemed on day 10 p.c., and in 1 of these 2 mice tachyzoites were present in the brain. In 6 mice, positive labelling for parasite antigens in infiltrating mononuclear cells were observed as well as tachyzoites and tissues cysts in brain samples analysed by IHC (see Fig. 4B). These *Neospora* antigen-positive cells were associated with moderate to severe lesions in all 5 of the mice. All of the mice sampled in Group 3 ($n=8$) gave positive *Neospora*-specific PCR results (see Table 2). (We were unable to collect samples from 2 mice in Group 3.)

Quantitative PCR

DNA extracted from brain samples of mice in Group 1 had a mean concentration of *Neospora* DNA of 0·011 pg/ng host genomic DNA (standard error of the mean (S.E.M.) $\pm$0·004 pg). Group 2 had a mean concentration of *Neospora* DNA of 0·042 pg/ng host genomic DNA ($\pm$0·014 pg). When the results from Groups 1 and 2 were compared by one-way ANOVA, it was shown that there was no statistical difference ($P=0·088$) between the amount of *Neospora* DNA per ng of host genomic DNA between the two groups. The results from Group 3 gave a mean concentration of *Neospora* DNA of 2·519 pg/ng host genomic DNA ($\pm$0·493 pg). When the results from Groups 1 and 2 were compared (one-way ANOVA) against those of Group 3, both groups that had previously been inoculated with either virulent or attenuated parasites showed significantly less ($P=0·001$) parasite DNA per ng of host genomic DNA than the control animals.

DISCUSSION

Previous work showed that it is possible to attenuate the virulence of *N. caninum* tachyzoites through prolonged passage in tissue culture (Long *et al*. 1998; Bartley *et al*. 2006). This present study shows that prior exposure to these attenuated parasites is sufficient to protect against a lethal challenge of virulent parasites. Mice exposed to low doses of virulent parasites were also protected against a lethal challenge, but demonstrated more severe pathology and a greater incidence of *Neospora*-DNA in the brain (86% positive) post-challenge, compared to the animals inoculated with attenuated parasites (56% *Neospora*-DNA positive).

The mortality rate in the control (Group 3) animals reached 100% by day 17 p.c., while all the vaccinated mice were protected against the lethal challenge and survived to the end of the experiment on day 28 p.c. Previous studies in Balb/c mice have shown mortality rates of up to 70% following a subcutaneous challenge with as few as $2·5\times10^5$ NC1 strain *N. caninum* tachyzoites (Lindsay *et al*. 1999) and up to 95% in mice following an i.p. challenge with 5×10^6 tachyzoites (Bartley *et al*. 2006). Lundén *et al*. (2002) previously showed that sublethal doses of live *N. caninum* tachyzoites were protective against a lethal challenge with the parasites; while, Lindsay *et al*. (1999) demonstrated protection against a lethal challenge with *Neospora* using live attenuated temperature sensitive mutant strains of the parasite. Ramamoorthy *et al*. (2006) have also demonstrated protection in Balb/c mice, through vaccination with γ-irradiated tachyzoites. This vaccine offered complete protection against parasite-induced mortality, whereas all the unvaccinated control animals challenged with 2×10^7 tachyzoites died by day 7 p.c. (Ramamoorthy *et al*. 2006)

The clinical symptoms seen in the mice during this study were similar to those described by Lindsay and Dubey (1989) and Lindsay *et al*. (1995), including rough coats, hunching, a reluctance to move, tottering gait and weight loss caused by a depressed appetite and dehydration. The naïve control mice that received the challenge of virulent parasites demonstrated a rapid onset of many of these

symptoms, which resulted in 100% (10/10) of the animals in the group being culled by day 17 p.c. Animals previously exposed to the parasite showed minimal clinical symptoms of infection, with limited ruffling of the coat, demonstrating that prior exposure to the parasite was sufficient to protect against the severe morbidity associated with *Neospora* challenge infections in Balb/c mice. Pinitkiatisakul *et al*. (2005) made similar observations, where mice immunized with recombinant NcSRS2 antigen then challenged i.p. with 1×10^7 live tachyzoites showed almost no morbidity; the non-immunized mice, however, showed neurological symptoms of neosporosis.

Previous studies have shown weight loss of 23% to 29·9% (Quinn *et al*. 2002; Bartley *et al*. 2006) following inoculation of Balb/c mice with *Neospora* parasites. In this present study a mean weight loss of 4·5 g (23·7%) occurred in primary challenge animals, while mice previously exposed to *Neospora* and then challenged continued to gain weight. This demonstrates weight loss to be a good indicator of severity of *N. caninum* infection in Balb/c mice, as the animals exhibiting the most severe morbidity also showed the greatest percentage weight loss.

Pathological changes were seen in the brains of many of the inoculated animals, indicating that prior exposure to the parasite does not always confer complete protection against infection with *N. caninum*. The pathology seen in the mice that initially received the attenuated parasite was milder than that seen in the mice inoculated with the virulent parasite, where a greater incidence of large foci of severe perivascular cuffing and necrosis was observed. The pathology observed in this study was comparable to that observed in previous experimental infections of mice (Lindsay *et al*. 1995; Long *et al*. 1998; Bartley *et al*. 2006). An interesting observation from this study was that there appeared to be no correlation between the presence of lesions in the brains of mice and the presence of overt clinical symptoms. Similar findings were reported by Ramamoorthy *et al*. (2006), where mice vaccinated with γ-irradiated tachyzoites showed no clinical signs of murine neosporosis, but at post-mortem neuropathology was observed.

Positive *Neospora*-specific ITS1 PCR results were seen in all the brain samples from mice in Group 3 from as early as day 10 p.c. These results are consistent with the findings of previous experiments, where positive PCR results were seen from day 7 p.i. following an i.p. inoculation with 5×10^6 NC1 tachyzoites (Bartley *et al*. 2006). Parasite DNA was detected in 86% of brain samples from mice in group 1 (which received the virulent parasites), but in only 56% of mice in Group 2 (which received the attenuated parasites). Previous work (Bartley *et al*. 2006) has shown that the attenuated parasites are still capable of dissemination and stage differentiation. The qPCR for *Neospora* DNA allowed reproducible, sensitive quantification of parasite burdens in infected mice. The qPCR results from this present study illustrated that there were significantly ($P < 0{\cdot}001$) lower levels of *Neospora* DNA in the brains of previously inoculated animals than was found in the brains of the control animals challenged with the virulent parasites. Prior exposure to the attenuated parasites resulted in 44% of the mice in Group 2 being PCR negative following challenge and lead to a significant reduction in the quantity of parasite DNA, compared to the control group. Although inoculation with virulent parasites also lead to a significant reduction in the quantity of parasite DNA post-challenge, only 14% of mice were PCR negative. These data would suggest that inoculation with the attenuated or virulent parasites is capable of reducing both the severity of infection and the parasite burden found in the brains of challenged animals.

The importance of the humoral immune response in mice during *N. caninum* infections was demonstrated by Eperon *et al*. (1999) who showed increased susceptibility to infection with the parasite in μMT B-cell deficient mice, compared to wild-type mice. In our study mice that were culled or died early in the experiment were seen to be producing *N. caninum*-specific IgM. Teixeira *et al*. (2005) also demonstrated production of *N. caninum*-specific IgM by day 7 p.c. followed by the production of IgG from about day 14 p.c., which is similar to the finding from our experiment, where all mice that survived to the end of the experiment seroconverted, producing high titres of *Neospora*-specific IgG. Though the role of the humoral immune response against *Neospora* has not been conclusively demonstrated; a likely role of antibodies would be in controlling the spread of the extracellular tachyzoite stage of the parasite (Innes *et al*. 2005). This hypothesis was further strengthened when Haldorson *et al*. (2006) showed that antibodies directed against the tachyzoite surface protein NcSRS2 inhibited the attachment and invasion of parasites *in vitro*. A further role of the B-cells may be in the activation of T-cells, since Teixeira *et al*. (2005) demonstrated that some $CD69^+$ B-cells from *N. caninum*-infected mice were also expressing CD80 and CD86, suggesting a role in T-cell activation.

The results from our study show that prior exposure to live attenuated tachyzoites not only protects against a lethal challenge of *N. caninum*, but also leads to milder pathology and a lower number of PCR-positive samples compared to inoculation and challenge with virulent parasites. Prior inoculation with either the attenuated or the virulent parasites significantly reduced morbidity, mortality and quantity of parasite DNA in brain tissue, compared to the control animals following challenge of all the groups with virulent parasites. Further work is

required to examine the immunological responses induced by these attenuated parasites and to determine whether vaccination with the attenuated tachyzoites can inhibit the transplacental transmission of the parasite.

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4 The development of immune responses in Balb/c mice following inoculation with attenuated or virulent *Neospora caninum* tachyzoites

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Parasite Immunology

4.1 Manuscripts main hypotheses

The main hypothesis that was tested in this manuscript is:

1 Are there quantifiable differences in the humoral and cell mediated immune responses generated in mice following a challenge with virulent or attenuated *Neospora* tachyzoites.

Where comparisons could be made (day 14 pi only) mice inoculated with either the virulent or the attenuated parasites had demonstrable anti-*Neospora* IgG titres. However, at the same time point (day 14 pi) higher levels of splenocyte proliferation and IFN-γ, IL-2, IL-4 and IL-10 production were seen in the mice receiving the attenuated parasites compared to those that received the virulent parasites. This experiment also demonstrated that prior exposure to the attenuated parasites not only protected against a challenge of $1x10^7$ virulent tachyzoites, which our previous work had shown to be highly lethal. But also lead to a reduction in the parasite burdens in the vaccinated / challenged animals, as demonstrated by both qPCR and IHC compared to the unvaccinated challenged mice.

Though this experiment clearly demonstrated that there were quantifiable differences in both the humoral and cell mediated immune responses between animals receiving attenuated or virulent parasites at day 14 pc, inter-group comparisons could only be made at one time point, as the initial challenge dose for the virulent parasites was too high. The dose ($5x10^6$) of parasites was chosen as it had previously been shown that that

number of parasites would produce a strong clinical response in Balb/c mice. Which when looking at a single time point, or at overall morbidity / mortality is fine. However for this experiment, as we were looking to compare responses over a time course experiment, then a lower initial dose of parasites should have been chosen. A dose of $2x10^6$ or $2.5x10^6$ tachyzoites would have probably been more appropriate. A lower parasite dose would have resulted in reduced mortality, allowing more detailed analysis of the immunological responses against the virulent *Neospora* parasites to be made over a longer period of time.

4.2 Author contributions

PMB, SEW, DB and EAI were involved in all aspects of the experimental design and planning of the experiments. All experiments were approved by Moredun Research Institutes experimental ethics committee.

PMB and SEW inoculated all the mice and were responsible for making clinical observations, daily morbidity scoring, weighing of individual mice and the culling of animals when needed. At post mortem examination PMB and SW removed the spleen intact for immunological examination and collected all of the tissue samples for PCR, qPCR and histological analysis and blood samples for serological screening.

PMB maintained the Vero cells and *Neospora* tachyzoites in tissue culture, enumerated the cells and tachyzoites using a Neubauer haemocytometer and prepared all of the inocula (Vero cells and tachyzoites) used during the experiments, PMB performed all immunological analysis, this include lymphocyte stimulation assays on mouse splenocytes, through the incorporation of ^{3}H thymidine, collected all of the splenocyte supernatant samples and analysed them by ELISA for the presence of the cytokines IL-2, IL-4, IL-10 and IFN-γ. PMB extracted the DNA from all of the tissue samples examined and performed all ITS1 PCR and NC5 qPCR analysis. PMB collated all of the data and prepared all of the graphs and tables presented in the manuscript.

SW separated serum from clotted blood samples and performed all serological testing (IgM and IgG) by IFAT, while SWM and DB performed all histological and immunohistological analysis and provided the images presented in the manuscript.

MN performed all of the statistical analysis on the mean group morbidity, mouse weight change, splenocyte proliferation, cytokine ELISA and qPCR data

PMB, SEW and EAI drafted the original manuscript with contributions from all other authors.

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The development of immune responses in Balb/c mice following inoculation with attenuated or virulent *Neospora caninum* tachyzoites

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SUMMARY

Balb/c mice were inoculated intraperitoneally (i.p.) with either 5×10^6 live virulent (group 1) or 5×10^6 live attenuated (group 2) tachyzoites, or Vero cells (group 3). Animals were killed at 0, 14, 28 and 42 days post-inoculation (p.i.), with the remaining mice receiving a lethal challenge on day 48 p.i. Serum, spleen and brain samples were collected post-mortem to examine humoral and cell-mediated immune responses as well as pathological lesions and to quantify parasite loads. On day 14 p.i. group 2 (attenuated) demonstrated statistically significant ($P < 0{\cdot}001$) lower levels of mean morbidity and weight loss, while also showing significantly ($P = 0{\cdot}01$) higher levels of splenocyte proliferation and IFN-γ production ($P = 0{\cdot}003$), compared to group 1 (virulent). Histology of brain samples showed milder lesions and a lower incidence of positive immunohistochemistry, demonstrating tachyzoites and tissue cysts, and statistically significant ($P = 0{\cdot}03$) lower mean burdens of parasite DNA in group 2 (attenuated) compared to group 1 (virulent). All mice in group 2 were protected following challenge on day 48 p.i. whereas naïve control mice succumbed to the challenge. No mice from group 1 (virulent) survived beyond day 24 p.i. so they were not included in the challenge.

Keywords *Balbc, cell mediated immunity,* Neospora caninum

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INTRODUCTION

Neospora caninum is an apicomplexan parasite which was first described in 1984, as an unidentified cyst forming sporozoan (1). *Neospora* is closely related to *Toxoplasma gondii*, has a world wide distribution and is considered to be a major cause of reproductive failure in cattle (2,3), and thus neosporosis has a major economic impact on the cattle (beef and dairy) industries (4).

A major route of transmission of *Neospora* in cattle is transplacentally from dam to foetus, though a primary infection may also occur following the ingestion of oocysts shed by dogs (a definitive host of the parasite) (5). Cattle infected with *Neospora* are 3–7 times more likely to abort than uninfected animals (6). However, McAllister *et al.* (7) have shown that cows exposed to *Neospora* prior to pregnancy are less likely to abort during a point source outbreak than cows with a primary infection. A recent study by Williams *et al.* (8) demonstrated protection against foetal death with an inoculation of live *N. caninum* tachyzoites, while Innes *et al.* (2) demonstrated that dams previously exposed to *N. caninum* inhibited the vertical transmission of the parasite following a challenge at mid gestation. All these results indicate that a level of protective immunity can be generated and that vaccination against neosporosis is a possibility.

Due to the intracellular nature of the parasite the predicted protective immune response would be predominantly a type 1 (Th1) cell-mediated immune (CMI) response with strong emphasis on lymphoproliferative responses as well as IFN-γ and IL-12 production. A number of experimental mouse models have shown this to be true (9–11). However, several recent studies have also suggested that a balanced Th1–Th2 response is required to limit a damaging host immune response, with great importance being placed on the production of IL-4 (12–15).

We have previously demonstrated that it is possible to attenuate the virulence of *Neospora* through prolonged *in vitro* passage (16) and that exposure to these attenuated parasites protects against a lethal challenge with the organism (17). This present study set out to investigate the development of humoral and CMI responses, parasite development and resultant pathology elicited by inoculation with either attenuated or virulent tachyzoites.

MATERIALS AND METHODS

Parasites and experimental inocula

Neospora caninum tachyzoites (NC1 strain) (18) were propagated as previously described (19). Briefly, the parasites were maintained by repeated passage in Vero cell monolayers in 25 cm^2 canted neck tissue culture flasks (Corning, NY, USA). Tachyzoites were harvested from the cell monolayers by disruption with a sterile cell scraper (Corning, NY, USA) and counted using a Neubauer haemocytometer then resuspended at a concentration of 5×10^7/mL or 1×10^8/mL (see Table 1). The control inoculum contained the equivalent number of Vero cells present in the parasite inoculum and was prepared in the same manner. All were inoculated intraperitoneally (i.p.) into mice within 1 h of their preparation in the laboratory, with each mouse receiving a 100 μL dose.

The *N. caninum* isolate used in this study is defined by the number of times (p.) it has been passaged (sub-cultured) *in vitro*. In previous studies (16,17) prolonged culture of *N. caninum* tachyzoites *in vitro* lead to the attenuation of virulence *in vivo*. The virulent parasites have been passaged continuously 34 times or 36 times, while the attenuated parasites have been passaged 76 times.

Experimental design

Female Balb/c mice approximately 12 weeks of age were randomly assigned into two groups each of 30 mice, group 1 (virulent) and group 2 (attenuated) and one group of 15 mice group 3 (Vero cells). The mice were individually identified by ear marking and given fresh water and rodent propriety mix *ad libitum*. The mice were inoculated i.p. with live *N. caninum* tachyzoites (Table 1) and observed daily. The morbidity of the animals was assessed according to a system agreed with the UK Home Office Inspectorate (16). Mice were randomly ascribed numbers at the start of the experiment and then killed in order, with six mice from group 1 and group 2 and three mice from group 3 to be killed on days 0, 14, 28 and 42 post-inoculation (p.i.). The remaining mice from all groups were administered with an i.p. challenge comprising live *N. caninum* tachyzoites on day 48 p.i. (see Table 1) and observed for a further 15 days. Mice were killed by CO_2 inhalation and at post-mortem examination blood was drawn from the heart to obtain serum, the spleen was excised and processed for immunological assays. The brain was then removed, cut longitudinally into two along the midline and one half was placed in 10% formal saline for histopathology and immunohistochemistry, while the other half was frozen and stored at −20°C prior to analysis by internal transcribed spacer (ITS1) PCR and SYBR green q-PCR.

Processing cells for immunological assays

At post-mortem examination the spleen was removed and placed in 5 mL wash buffer [Hanks balanced saline solution supplemented with 2% heat inactivated foetal bovine serum (ΔH-FBS) (Labtech, Mornington, Australia), 100 IU/mL penicillin and 100 μg/mL streptomycin (Northumbria Biologicals, Cramlington, UK)]. In the laboratory the spleen was placed in a sterile Petri dish and slowly injected with 10 mL of wash buffer, using 2 × 5mL syringes and 21G needles, causing the spleen to expand and rupture to release splenocytes, which were then decanted into a sterile universal and centrifuged at 280 ***g*** for 5 min. The splenocytes were washed × 2 in 10 mL wash buffer by repeated centrifugation, counted using a Neubauer haemocytometer and resuspended at a concentration of 2×10^6 cells/mL in cell culture media (CCM) (Iscoves modified Dulbecco's

Table 1 Experimental design

Group	Inoculation[a] (day 0 p.i.)	Days p.i./no. of mice (no. at PM)				Challenge[a] (day 48 p.i.)	Day p.i.
		0	14	28	42		54–63
1	5×10^6 NC1 (p. 34) (virulent)	6 (6)	6 (4)	6 (0)	6 (0)	1×10^7 NC1 (p. 36) (virulent)	6 (0)
2	5×10^6 NC1 (p. 76) (attenuated)	6 (6)	6 (6)	6 (5)	6 (5)	1×10^7 NC1 (p. 36) (virulent)	6 (6)
3	$4{\cdot}35 \times 10^5$ Vero cells	3 (3)	3 (3)	3 (3)	3 (3)	1×10^7 NC1 (p. 36) (virulent)	3 (3)

[a](p. 34), passage number 34; (p. 76), passage number 76; (p. 36), passage number 36.
p.i., post-inoculation; NC, *Neospora caninum*.

medium) supplemented with 10% ΔH-FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin).

Cell proliferation assays

Equal volumes of splenocytes (2×10^6/mL) (see above) and antigens were added in triplicate to 96-well round-bottomed tissue culture plates (Nunc, Roskilde, Denmark). The *N. caninum* antigen (NCA) consisted of a water-soluble lysate of tissue culture derived *N. caninum* tachyzoites, prepared as previously described by Innes *et al.* (2). The NCA was used in culture at a final concentration of 2·5 μg/mL. Cells were also cultured with the T-cell mitogen Con A at a final concentration of 2·5 μg/mL, which was used as a positive control, while Vero cell antigen at a final concentration of 1 μg/mL was used to ensure the immune responses observed were *Neospora* specific and not against the contaminating Vero cells. The CCM was used as a negative control to determine the background level of cell proliferation. Plates were placed in a 37°C incubator with a humidified 5% CO_2 atmosphere for 4 days. Cells were pulsed for the final 18 h with 18·5 KBq ^{3}H thymidine (Amersham Biosciences, Bucks, UK) per well, then harvested onto fibreglass filters (Wallac, Turku, Finland), the cell associated radioactivity was determined using a microbeta Trilux liquid scintillation counter (Perkin Elmer, Wellesley, MA, USA).

A duplicate assay was set up for each sample; cell-free supernatants were harvested after 4 days and stored at −20°C prior to analysis for levels of cytokine production.

Enzyme-linked immunosorbent assays for the detection of murine cytokines

Cell-free supernatants (see above) were examined for the presence of IL-2, IL-4, IL-10 and IFN-γ using commercially available ELISA kits (Diaclone, Besançon, France). Assays were performed as per manufacturers instructions. Standard curves were generated for each cytokine by plotting absorbance reading (optical density (OD) value) against known standard cytokine concentrations. The OD value of each experimental sample was extrapolated against these standard curves to determine experimental cytokine concentrations.

Serology

Serum collected post-mortem was processed as described by Bartley *et al.* (17) and tested for the presence of *Neospora*-specific IgM and IgG by indirect fluorescent antibody test using the procedure described by Bartley *et al.* (16). The endpoint was determined as the final concentration of sera that demonstrated whole tachyzoites fluorescence (20).

ITS1 and SYBR Green q-PCR

The nested PCR used to detect *Neospora*-specific ITS1 DNA (21) was performed using the method previously described by Buxton *et al.* (22). This produced a band of 297 bp, when the amplification products were analysed using 1·8% agarose gel electrophoresis, stained using ethidium bromide and visualized under ultraviolet light. Samples that tested positive by the ITS1 PCR were tested using the qPCR for the NC5 sequence of *N. caninum*, which was performed using the primers previously described by Collantes-Fernandez *et al.* (23) and the method, standards and amplification conditions previously described by Bartley *et al.* (17).

Histology and immunohistochemistry

After 2–4 weeks fixation each half brain was sliced coronally to give blocks through the anterior cerebrum, midcerebrum at the level of the thalamus, the posterior cerebrum at the level of the hippocampus, the midbrain, the cerebellum and the pons. These samples were processed through graded alcohols to paraffin wax. Sections 4-μm thick were cut and stained with haematoxylin and eosin as described by Lunden *et al.* (12). Pathological findings in the brain were scored as follows: 0 = no changes; 1 = pathological changes present (mild); 2 = pathological changes moderate (often only involving one or two sections); 3 = pathological changes severe (usually also involving all sections). Serial sections were cut and subjected to an immunohistochemical method to demonstrate both *N. caninum* bradyzoites in tissue cysts and tachyzoites, as previously described by Lunden *et al.* (12).

Statistical analysis

The Kruskal–Wallis one-way analysis of variance was applied to evaluate the average morbidity score data of individual mice from day 0 to 14 p.i, with a nonparametric two-sided Mann–Whitney test being used to compare the data from group 1 (virulent) and group 2 (attenuated) mice. A repeated measures model was fitted to the body weight data, using a first order autoregressive correlation structure with *P*-values estimated using modified *F*-statistics (24). Multiple comparisons with Bonferonni approximation were used to compare the body weights of group 1 (virulent) and group 2 (attenuated) mice. The CMI responses from the proliferation assays were evaluated using a linear mixed model with *P*-values estimated using modified *F*-statistics (24). For the cytokine (IFN-γ, IL-2, IL-4 and IL-10) and humoral (IgG and IgM) responses, data were categorized into different categories and two-

sided Fisher's exact test for an $m \times n$ contingency table was carried out. A Student's unpaired two-sample *t*-test (two-tailed) allowing for unequal sample variances was used to compare the quantitative PCR data from group 1 (virulent) and group 2 (attenuated). All statistical analyses were undertaken using the GenStat 10th edition statistical package (VSNI, Hemel Hempstead, UK) except the Fisher's exact test, which was implemented in the statistical package R, version 2.6.0. Where applicable, results are presented as mean values along with the standard error of mean values.

RESULTS

Mice from group 1 (virulent) were killed at different time points due to high morbidity scores and as a result no mice from group 1 survived beyond day 24 p.i., and samples could not always be collected from all animals. Samples from animals that were killed/died before day 14 p.i. were compared only by PCR and histology. From day 14 p.i. onwards animals that were killed/died were not included in the analyses as no group comparison could be made.

Clinical observations

Three mice from group 1 (virulent) were incorrectly inoculated, as a result, these animals showed no clinical symptoms, generated no humoral or CMI responses, were negative by ITS1 PCR and demonstrated no pathology. As a consequence, the results from these animals were removed from all data sets.

Group 1 mice (virulent) demonstrated ruffled coats from day 1 p.i., other symptoms including a reluctance to move, tottering gait, a stiff stary coat and weight loss were observed from day 4 p.i., resulting in an increase in the group mean morbidity score (see Figures 1 and 2). Group 2 mice (attenuated) demonstrated very mild transient symptoms of murine neosporosis, with a ruffled coat being observed from day 2 p.i., all mice in group 2 returned to being clinically normal by day 6 p.i. (see Figure 1). No weight loss was seen in group 2 following the inoculation (see Figure 2). When the morbidity data from group 1 and group 2 were compared, group 1 demonstrated a statistically significant ($P < 0{\cdot}001$) higher mean morbidity score than group 2 between days 5 and 14 p.i. When the weights of the mice in group 1 and group 2 were compared; group 1 demonstrated statistically significant ($P < 0{\cdot}001$) levels of mean weight loss between day 6 p.i. and day 14 p.i. (see Figure 2). Group 2 continued to show no clinical symptoms and demonstrated no weight loss between day 28 p.i. and 42 p.i. All mice in group 2 showed a slightly ruffled coat from day 1 post-challenge (p.c.) (day 49 p.i.), then returned to being clinically normal on day 4 p.c. (day 52 p.i.) (data not shown). Group 3 mice (Vero cells) showed no clinical symptoms (see Figure 1) nor demonstrated any weight loss between day 0 p.i. and day 48 p.i. (see Figure 2). Following the challenge on day 48 p.i. all the mice in group 3 showed a ruffled coat from day 1 p.c. (day 49 p.i.), symptoms progressed to include a stiff stary coat, hunching, a reluctance to move, tottering gait and weight loss, which resulted in all the mice in group 3 being culled on day 6 p.c. (day 54 p.i.) (data not shown).

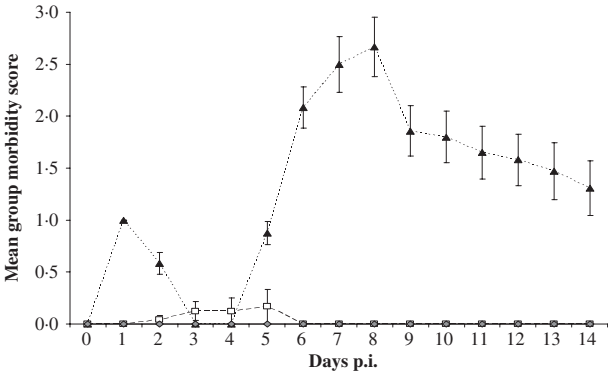

Figure 1 Mean group morbidity score following i.p. inoculation with live *N. caninum* tachyzoites. Group 1, -▲- 5×10^6 NC1 (p. 34) (virulent). Group 2, -□- 5×10^6 NC1 (p. 76) (attenuated). Group 3 -●- $4{\cdot}35 \times 10^5$ Vero cells. Error bars ± SEM.

Cell-mediated immune response

The results from the proliferation assays are expressed as stimulation indices (S.I.), these are calculated by dividing the mean counts per minutes (cpm) from the NCA stimulated wells by the mean cpm from the CCM stimulated wells.

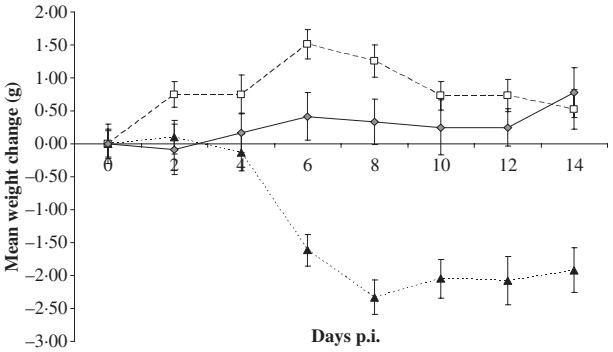

Figure 2 Mean group weight change (g) following i.p. inoculation with live *N. caninum* tachyzoites. Group 1, -▲- 5×10^6 NC1 (p. 34) (virulent). Group 2, -□- 5×10^6 NC1 (p. 76) (attenuated). Group 3 -●- $4{\cdot}35 \times 10^5$ Vero cells. Error bars ± SEM.

Cell proliferation

On day 14 p.i., group 1 mice (virulent) demonstrated antigen-specific responses to NCA, with a mean S.I. of 12·00 (±5·19), while group 2 mice (attenuated) demonstrated a mean NCA specific S.I. of 18·84 (±1·23). When the proliferation data from group 1 and group 2 on day 14 p.i. were compared; group 2 was seen to produce a statistically significant (P = 0·01) greater level of NCA specific proliferation than group 1 (see Figure 3). Group 2 mice maintained elevated levels of antigen specific proliferation throughout the course of the experiment on days 28 and 42 p.i. and on day 15 p.c. (day 63 p.i.) group 2 demonstrated a mean NCA-specific S.I. 6·29 (±2·01) (see Figure 3). Group 3 mice (Vero cells) demonstrated a low background level of cell proliferation on days 14, 28 and 42 p.i. (S.I. 1·49 ± 0·14, 1·51 ± 0·31 and 0·62 ± 0·03 respectively). Following the challenge with virulent *N. caninum* parasites on day 48 p.i, a mean NCA-specific cell proliferation S.I. 9·54 (±7·84) was seen in group 3 mice on day 54 p.i. (day 6 p.c.) (see Figure 3). Low background levels of cell proliferation to NCA were observed in all groups on day 0 p.i. (see Figure 3).

Cytokine responses

Supernatants from splenocytes stimulated for 4 days with NCA were tested for the production of IFN-γ, IL-2, IL-4 and IL-10. On day 14 p.i. NCA supernatants from group 1 (virulent) and group 2 (attenuated) showed production of IFN-γ, IL-2, IL-4 and IL-10 (see Figure 4a–d), when the quantities of cytokine produced in groups 1 and 2 were compared, it was seen that group 2 produced statistically significant (P = 0·003) more IFN-γ than group 1. However, although the levels of IL-2, IL-4 and IL-10 produced were higher in group 2, these differences were not statistically significant (P = 0·085, P = 0·186 and P = 0·172 respectively) (see Figure 4a–d). In group 2 mean levels of IFN-γ peaked on day 14 p.i., then decreased on days 28 and 42 p.i. (see Figure 4a); mean levels of IL-2 peaked on day 42 p.i. (see Figure 4b), while on days 28 and 42 p.i. mean levels of IL-4 and IL-10 remained low but detectable; however, following the virulent challenge administered on day 48 mean levels of IL-4 and IL-10 increased from baseline (see Figure 4c and d). Group 3 (Vero cells) produced very low or undetectable levels of IFN-γ, IL-2, IL-4 and IL-10 on days 14, 28 and 42 p.i. Following the challenge with virulent *N. caninum* parasites levels of all the cytokines tested in group 3 increased from baseline (see Figure 4a–d).

On day 0 p.i. IFN-γ, IL-2, IL-4 and IL-10 was produced in low levels or in levels that were below the detection threshold of the ELISA (see Figure 4a–d) in all three groups.

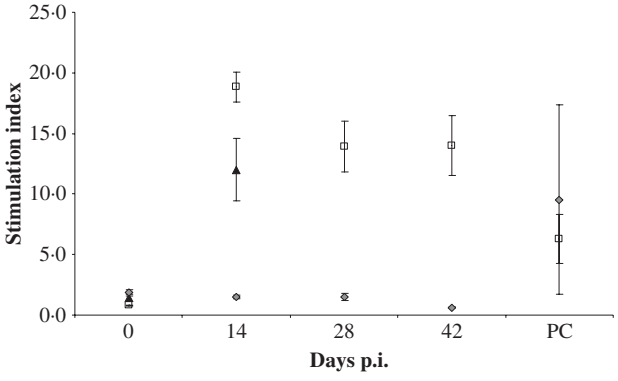

Figure 3 Mean proliferation results of splenocytes following stimulation with NCA (expressed as S. I.). Group 1, -▲- 5×10^6 NC1 (p.34) (virulent). Group 2, -□- 5×10^6 NC1 (p.76) (attenuated). Group 3 -●- $4{\cdot}35 \times 10^5$ Vero cells, PC = post-challenge, error bars ± SEM.

Serology

On day 14 p.i. group 1 mice (virulent) showed IgG titres of 1/64–1/128. Group 2 mice (attenuated) showed IgG titres of 1/64–1/256. When the levels of IgG from group 1 and group 2 on day 14 p.i., were compared it was seen that the mice in group 1 generally had lower IgG titres than group 2, though this difference was not statistically significant (P = 0·065). Group 2 mice demonstrated IgG titres of 1/256–1/512 on day 28 p.i., on day 42 p.i. all group 2 mice tested had IgG titres of 1/1024, on day 15 p.c. (day 63 p.i.) the IgG titres were between 1/2048 and 1/4096. Group 3 mice (Vero cells) showed IgG titres of <1/16 on days 14, 28 and 42 p.i. Following the challenge with virulent parasites on day 48 p.i., no anti-*Neospora* antibodies were detectable in samples collected from group 3 mice on day 6 p.c. (day 54 p.i.). On day 0 p.i. IgG titres of <1/16–1/16 were detected for all three groups.

Titres of IgM between <1/16–1/64 were detected from all three groups of mice at all of the time points tested, giving no statistically significant differences between any of the groups at any of the time points tested.

ITS1 and SYBR green PCR

In group 1 (virulent), positive results were seen in all (8/8) of brain samples tested between day 8 and 14 p.i. The results from the qPCR show that these mice had a mean value of 1·19 (±0·44) pg of *Neospora* DNA per ng of host genomic DNA. In group 2 (attenuated) positive

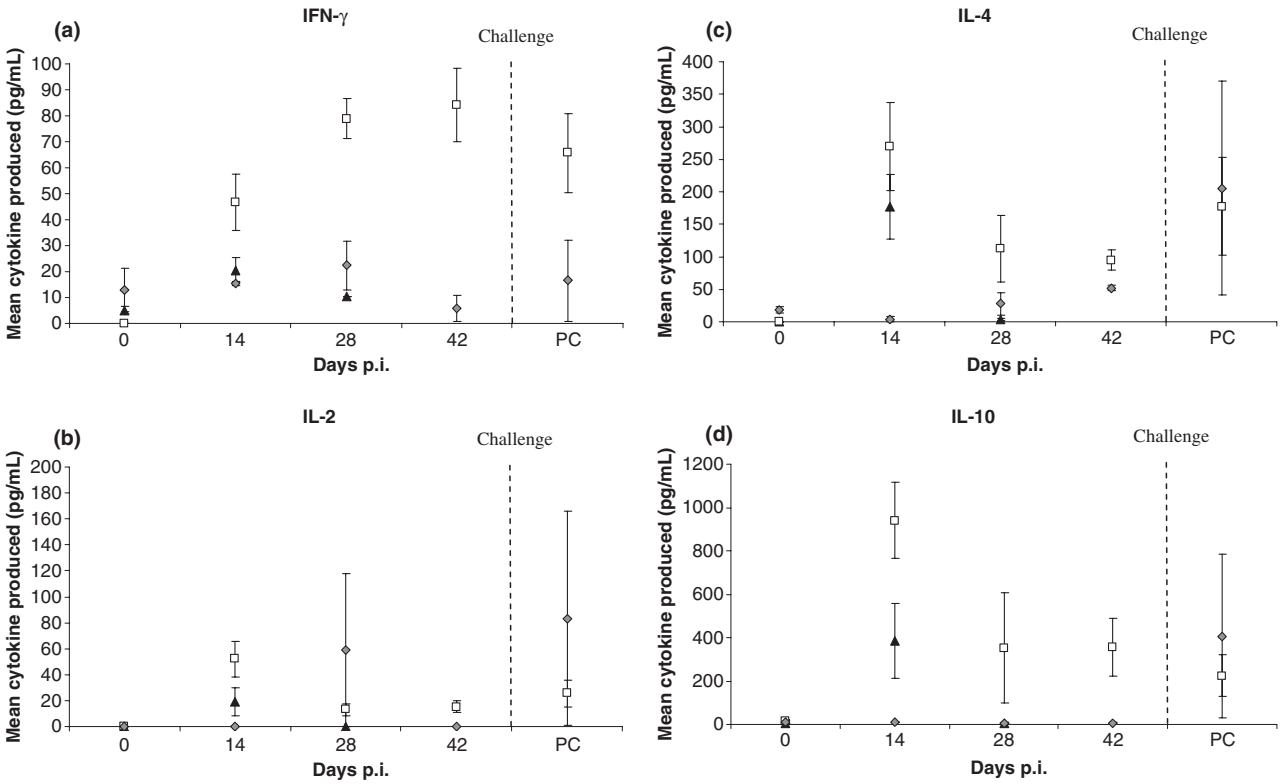

Figure 4 Cytokine production following stimulation with NCA. Group 1, -▲- 5×10^6 NC1 (p. 34) (virulent). Group 2, -□- 5×10^6 NC1 (p. 76) (attenuated). Group 3 -●- $4{\cdot}35 \times 10^5$ Vero cells, PC = post-challenge, error bars ± SEM.

results were seen in (2/6) of brain samples on days 13 and 14 p.i. The qPCR showed that the samples that tested positive by ITS1 PCR had a mean of 0·005 (±0·004) pg of *Neospora* DNA per ng of host genomic DNA. When the qPCR results from group 1 and group 2 were compared, group 1 was seen to have a statistically significant ($P = 0{\cdot}03$) higher parasite burden than group 2 between days 8 and 14 p.i. In group 2 on day 28 p.i. positive ITS1 PCR samples were seen in 2/5 samples, (0·36 ± 0·34 pg of *Neospora* DNA per ng of host genomic DNA), whilst on day 42 p.i. 1/4 samples were positive (0·006 ± 0·02 pg of *Neospora* DNA per ng of host genomic DNA). In group 2 following the challenge with virulent *N. caninum* parasites, 2/6 samples tested positive on day 15 p.c. (day 63 p.i.). The qPCR results from these mice demonstrated a mean parasite burden of 0·087 ± 0·027 pg per ng host DNA. All the samples tested from mice in group 3 (Vero cells) on days 14, 28 and 42 p.i. were negative by ITS1 PCR. Following the virulent challenge all three remaining mice in group 3 were PCR positive on day 6 p.c. (day 54 p.i.), where a mean of 0·47 (±0·22) pg of *Neospora* DNA per ng host DNA was seen. All the samples tested from all three groups were negative by ITS1 PCR on day 0 p.i.

Pathology and immunohistochemistry

In group 1 (virulent) four mice culled on days 9 and 13 p.i. demonstrated mild pathological changes (see Table 2), this composed of, in each case, one small clearly delineated focus of necrosis infiltrated by a mixed population of cells, comprised chiefly of microglia and mononuclear inflammatory cells and also containing nuclear debris. Immunohistochemistry allowed identification of *Neospora* tachyzoites in the periphery of the lesion with some also in the surrounding neuropil (Figure 5). A small tissue cyst was present adjacent to one focus. In those mice sampled on day 14 p.i. there were moderate changes made up typically of several foci of inflammation some small and clearly delineated and some larger, more diffuse and less intense. Cells were chiefly microglia and mononuclear infiltrating cells. Associated capillaries were prominent due to a combination of pericyte proliferation and the presence of infiltrating inflammatory cells (Figure 6). *Neospora* tachyzoites were present in variable numbers in six of the

Table 2 Individual pathology scores

	Individual pathology scores								
Days p.i.	0–6[a]	7–13[a]	14–20[a]	21–27[a]	28–34[a]	35–41[a]	42[a]		Day 15 p.c.[a]
Group 1	0, 0, 0, 0, 0, 0	1, 1, 1, 1	2, 2, 2, 2, 2, 2	3, 3, 3, 3, 3, 3	–	–	–	Challenge	–
Group 2	0, 0, 0, 0, 0, 0	1	2, 0, 0, 1, 0	–	2, 0, 1, 3, 0	0	2, 2, 2, 1, 2		1, 2, 0, 0, 1, 1
Group 3	0, 0, 0	–	0, 0, 0	–	0, 0, 0	–	0, 0, 0		–[b]

[a]No statistical differences observed between groups; [b]Mice culled on day 6 p.c.
p.c., post-challenge.

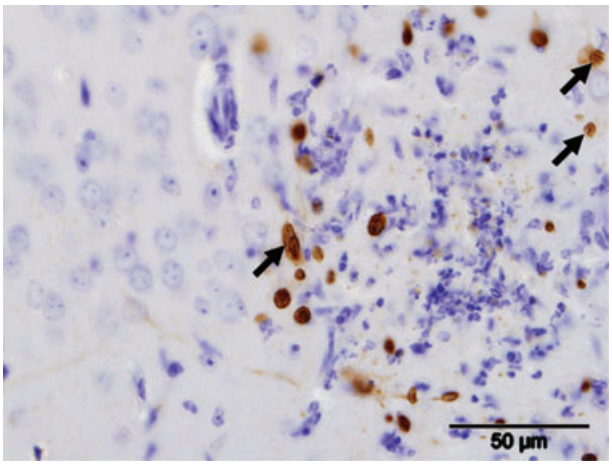

Figure 5 A small focus of necrosis in the cerebrum of a mouse in group 1 (virulent) killed on day 9 p.i. showing dividing *N. caninum* tachyzoites (arrows). (Immunohistochemistry).

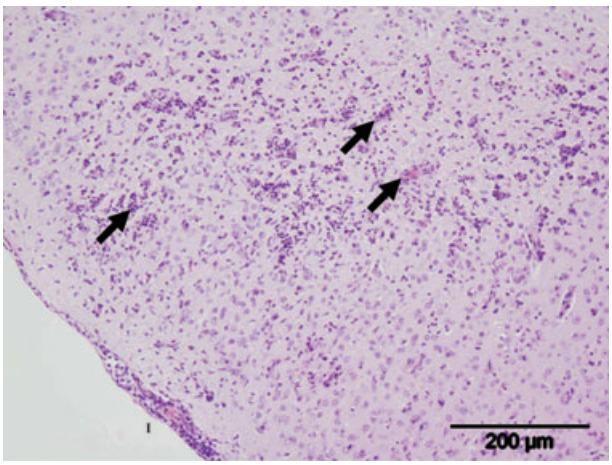

Figure 6 A focus of inflammation in the neuropil of the cerebrum (arrows) with associated inflammation in the adjacent meninges (I) of a mouse in group 1 (virulent) killed on day 14 p.i. (haematoxylin and eosin).

eight mice, while bradyzoites in tissue cysts were present in three of the eight mice tested between days 9 and 14 p.i. In group 2 (attenuated). histopathological changes were present to a variable extent on days 13 and 14 p.i., of the six mice sampled, three had no lesions, two had mild changes and one had moderate changes, of the five mice sampled on day 28 p.i. two had no changes, one had mild, one had moderate and one had severe changes. In all cases there was lymphoid cuffing and associated microgliosis. *Neospora* antigen was demonstrated only in this last case and it consisted of traces of particulate antigen. On day 42 p.i. mild pathological changes were seen in one of the five mice sampled while moderate changes were present in the other four (see Table 2). In one of which there were traces of particulate antigen and in another there were a few scattered tachyzoites. Following the challenge with virulent parasites on day 48 p.i. all the mice in group 2 survived to the end of the experiment on day 15 p.c. (day 63 p.i.). Samples taken from the mice at post-mortem showed that two had no pathological changes, three showed mild pathological changes and one mouse showed moderate pathological changes. The only IHC positive sample seen at this time was in one mouse with mild lesions, in which there were a few scattered tachyzoites associated with inflammation. The only tissue cyst seen in group 2 was in one mouse killed on day 14 p.i. Group 3 showed no pathological changes in any of the brains from mice sampled on days 14, 28 and 42 p.i. A small focus of nonsuppurative inflammation was present in the cerebellum of one of the three mice in group 3 sampled on day 6 p.c. (day 54 p.i.), numerous tachyzoites were also visible within this lesion by IHC. No pathological changes were seen in any of the brain samples taken from all three groups on day 0 p.i.

DISCUSSION

The results from this study show clear differences in the development of host immune responses and lesions in mice inoculated with attenuated or virulent *N. caninum* tachyzoites. Mice inoculated with the attenuated parasite demonstrated statistically significant reduced clinical symptoms ($P < 0{\cdot}001$), higher levels of *Neospora* specific

cell proliferation ($P = 0{\cdot}01$) and IFN-γ production ($P = 0{\cdot}003$). Group 2 also produced higher levels of IL-2, IL-4 and IL-10 production and stronger antibody (IgG) responses than the mice that were inoculated with the virulent parasites. The mice inoculated with the attenuated parasites also demonstrated milder pathological changes, reduced evidence of parasite in brain samples, leading to lower parasite burdens as demonstrated by both IHC and qPCR, when compared with the mice inoculated with the virulent parasite. However, as we were only able make comparisons between the mice inoculated with either the virulent or attenuated parasites at a single time point (day 14 p.i.); we must also acknowledge the fact that the kinetics of infection of the virulent and attenuated parasites may differ, which may contribute to differences in morbidity, mortality as well as the immune responses observed at this time point.

The milder clinical symptoms seen in the mice inoculated with the attenuated parasites, compared to those in the mice inoculated with the virulent parasites were similar to the results of previous studies (16,25). The attenuation of parasites through prolonged *in vitro* passage, (16) has also been shown for *Babesia bigemina*, where microaerophilous stationary phase cultures repeatedly passaged *in vitro* elicited no signs of clinical babesiosis following inoculation into naïve calves (26). Attenuation of *Leishmania* spp. has also been achieved through the *in vitro* passage of mid- to late-log phase promastigotes, cultured in the presence of gentamicin (27). Inoculation into susceptible hosts with these attenuated parasites resulted in reduced morbidity and protection against a subsequent lethal challenge with wild-type virulent parasites.

This present study shows a strong stimulation of CMI responses, involving *Neospora*-specific lymphoproliferation of splenocytes, following *in vitro* stimulation with NCA and the production of the cytokines IFN-γ and IL-2, as well as the production of IL-4 and IL-10. Due to the intracellular nature of *N. caninum*, the protective immune response is likely to involve cell-mediated immunity and studies have emphasized the importance of IL-12 and IFN-γ (9–11). Little is currently known about the induction of IFN-γ responses to *Neospora* in mice during the early (acute) phase of infection. However, work carried out on the closely related parasite *T. gondii* has shown that the parasites can directly induce macrophages to produce TNF-α and IL-12, which in turn induces NK cells to produce IFN-γ (28). This increase in IL-12 has been detected in spleen and peritoneal cells as early as 2 days post-infection (29). During intracellular infections with *T. gondii* and *L. major*, IL-12 and IFN-γ have been shown to mediate the development of antigen-specific Th1 cells which secrete the effector cytokines IL-2 and TNF-β. These activated Th1 cells promote cytolytic responses and antibody class switching towards antibody-dependent cell killing (30). Both IL-12 and IFN-γ have been shown to be critical in protection against *N. caninum* in previous studies, where IL-12 and IFN-γ knockout (IL-12 KO and IFN-γ KO) mice and the *in vivo* depletion of either IL-12 or IFN-γ using monoclonal antibodies lead to increased susceptibility to the parasite (10,11). Recent studies in cattle have also demonstrated that; NK cells play a key role in early innate immune responses to *Neospora* through the IL-12 independent production of IFN-γ (31). Klevar *et al.* (32) have also demonstrated that NK cells and $CD8^+$ T-cells are important in producing IFN-γ in the early stages following *Neospora* infection in calves.

Our findings showed that the mice generating the strongest IFN-γ response also demonstrated the strongest IL-10 response and these mice tended to show the mildest pathological changes, with reduced parasite loads. This finding is supported by a recent study by Nishikawa *et al.* (14) who demonstrated that both Th1/Th2 type responses are crucial in controlling a *Neospora* infection and limiting the immunopathology caused by an excessive pro-inflammatory response. Long *et al.* (10) demonstrated increased susceptibility in mice producing high levels of the Th2 type cytokine IL-4 following an i.p. inoculation with live *Neospora* tachyzoites. In addition, Baszler *et al.* (9) demonstrated that a bias towards a Th2 response lead to increased disease and severity of brain lesions in mice inoculated with *Neospora* antigen and Freund's complete adjuvant then challenged with live tachyzoites. Our findings would suggest that while the Th1 cytokine IFN-γ may be important in controlling the spread of the parasite, Th2 cytokines including IL-10 may play a role in limiting the pathological changes caused by an excessive pro-inflammatory response.

The importance of the humoral response during murine neosporosis was demonstrated by Eperon *et al.* (33) who showed increased susceptibility to *N. caninum* in μMT B-cell deficient mice, compared to wild type C57BL/6 mice. The humoral response described here, showed an increase in IgG over time. Similar findings were observed by Teixeira *et al.* (34) who described that an ip inoculation of mice with live tachyzoites induced a rapid increase in the numbers of peritoneal and splenic B-cells, preceding a rise in serum levels of *Neospora*-specific immunoglobulins. A suggested function of antibodies is in clearing the extracellular tachyzoites (35), this idea is supported by work from Haldorson *et al.* (36) who demonstrated that antibodies directed against the *Neospora* tachyzoite surface protein NCSRS2 significantly blocked the invasion of the parasite into host cells. B-cells however not only play a central role in antibody production and regulation, but can also act as

antigen presenting cells and are capable of secreting Th1- and Th2-type cytokines (37).

The pathological changes observed in mice inoculated with *N. caninum* in this study was comparable to that of previous *in vivo* experiments (16,17). The mice that were inoculated with the attenuated parasites showed a much lesser incidence and severity of pathological changes, compared to the mice inoculated with the virulent parasites. Similar findings were seen in experiments where mice immunized with live temperature sensitive mutant NCts-8 parasites (38), live γ-irradiated parasites (39) and bacterially expressed recombinant NCMIC3 (40) or NcSRS2 (41) not only demonstrated protection against a lethal challenge with *N. caninum*, but also showed reduced morbidity and milder pathological changes compared to naïve mice challenged with virulent *N. caninum* parasites.

Lesser parasite burdens were observed in the brains of mice inoculated with the attenuated parasites, when compared to the mice that were inoculated with the virulent parasites confirming results from previous studies (16,17). In addition within group 2, (attenuated) substantially more mice were PCR negative compared to group 1 (virulent), suggesting a very low parasite burden in the mice inoculated with the attenuated parasites. Interestingly, there was no correlation between neuropathology and overt clinical symptoms of murine neosporosis, as one mouse that received the attenuated parasites showed severe pathological changes but had appeared clinically normal; Ramamoorthy *et al.* (39) made similar observations in mice inoculated with γ-irradiated parasites which were then challenged.

The results from this study show that inoculation with live attenuated parasites generates a protective immune response against *Neospora* and that this protection involves both the humoral (IgG) and CMI responses. While the lymphoproliferative and IFN-γ responses are critical in controlling dissemination of the parasite, IL-10 may help to limit the immunopathology caused by the Th1 cytokines. This suggests that a balance is needed between the immune responses that are important in controlling infection and those involved in reducing immunopathology. Mice inoculated with the attenuated parasites also showed reduced incidence and burdens of parasite DNA in the CNS, compared to mice inoculated with virulent parasites.

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5 Selection of *Neospora caninum* antigens stimulating bovine CD4+ve T cell responses through immuno-potency screening and proteomic approaches

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Veterinary Research

5.1 Manuscripts main hypotheses

The main hypotheses that were tested in this manuscript are:

1. Can size-exclusion HPLC be used to fractionate *Neospora caninum* tachyzoite water soluble antigens (NcWSA)?
2. Can T-cells ($CD4^+$) derived from experimentally infected cattle be used to screen for immunologically reactive proteins from the HPLC separated fractions of NcWSA?
3. Can LC-ESI-MS/MS (liquid chromatography electrospray ionisation tandem mass spectrometry) identify individual immunoreactive proteins?

The results from this experiment shows that HPLC effectively fractionates NcWSA and that immune screening using $CD4^+$ T-cells from experimentally infected cattle identified 3 highly immunoreactive fractions (fractions 3, 4 and 5). Phenotypic analysis of the responding T-cells clarified that $CD4^+$ are the predominant cells involved in the proliferative response, with a minor involvement of $CD8^+$ and γδ T-cells. The LC-ESI-MS/MS analysis of the individual fractions identified a number of *Neospora* surface proteins including SAG1, SRS2 as well as dense granule proteins GRA2 and GRA7 and microneme proteins MIC3 and MIC11. Along with the *Neospora* peptides, 17 *Toxoplasma gondii* homologues were also identified. Many of the *Neospora* peptides recognised by this assay have known functions in either parasite attachment or host cell

invasion, meaning that these proteins are often exposed to the host immune response and are highly antigenic.

Though this experiment identified a number of highly immunogenic parasite antigens, it failed to produce or identify any novel antigens. Though NcMIC11 had previously been identified, it had not been shown to be immunologically reactive. The HPLC method of fractionation is also too crude, as it only separates by peptide size, meaning that each fraction still contains large numbers of different peptides. It also must be remembered that this paper only demonstrates that peptides are immuno-reactive, not that they immuno-protective. Further studies would be required, either in cattle or mice to actually demonstrate a role for each of the peptides in a protective immune response.

For a clearer indication of the roles of individual peptides then each HPLC fraction should be further separated i.e. by 2D electrophoresis, then individual spots could be tested for their immunoreacitivity then characterised by LC-ESI-MS/MS.

5.2 Author contributions

PMB, MSR, NFI, FK and EAI were involved in all aspects of the experimental design and planning of the experiments. All experiments were approved by Moredun Research Institutes experimental ethics committee.

PMB and MSR inoculated all of the cattle and were responsible for routinely collecting blood samples and for the generation of the CD4+ T-cell lines and γ-irradiated autologous APC's used to screen for immune-reactive peptides.

PMB maintained the Vero cells and *Neospora* tachyzoites in tissue culture. PMB enumerated the cells and tachyzoites using a Neubauer haemocytometer and prepared all of the inocula (Vero cells and tachyzoites) used during the experiment. PMB and MSR prepared the water soluble tachyzoite antigen used in the HPLC fractionation and LC ESI MS/MS analysis. PMB, MSR and ECF were involved in the lymphocyte stimulation assays using the fractionated antigens.

NFI carried all HPLC /LC ESI MS/MS analysis of the *Neospora* water soluble antigen fraction

PMB carried out all of the MASCOT analysis on the immune-reactive fractions. MSR prepared all of the figures and PMB prepared all of the tables presented in the manuscript

MSR, PMB, NFI, FK and EAI drafted the original manuscript with contributions from all other authors.

VETERINARY RESEARCH

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Selection of *Neospora caninum* antigens stimulating bovine CD4^{+ve} T cell responses through immuno-potency screening and proteomic approaches

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Abstract

Neospora caninum is recognised worldwide as a major cause of bovine infectious abortion. There is a real need to develop effective strategies to control infection during pregnancy which may lead to either abortion or congenital transmission. Due to the intracellular nature of the parasite, cell-mediated immune (CMI) responses involving CD4^{+ve}, CD8^{+ve}, γ/δ TCR^{+ve} T cells and NK cells, as well as production of IFN-γ, are thought to be important for protective immunity. In this study we applied a combination of proteomic and immunological approaches to identify antigens of *N. caninum* that are recognized by CD4^{+ve} T cell lines derived from infected cattle. Initially, *N. caninum* tachyzoite Water Soluble Antigens (NcWSA) were fractionated by size-exclusion HPLC and then screened for immune-potency using CD4^{+ve} T cell lines. LC-ESI-MS/MS (liquid chromatography electrospray ionisation tandem mass spectrometry) was employed to catalogue and identify the proteins comprising three immunologically selected fractions and led to the identification of six *N. caninum* target proteins as well as sixteen functional orthologues of *Toxoplasma gondii*. This approach allows the screening of biologically reactive antigenic fractions by the immune cells responsible for protection (such as bovine CD4^{+ve} cells) and the subsequent identification of the stimulating components using tandem mass spectrometry.

Introduction

Neospora caninum is a protozoan parasite, closely related to *Toxoplasma gondii*, which has emerged as a major cause of reproductive failure in cattle worldwide [1,2]. The parasite is now recognised as the most commonly diagnosed cause of abortion in areas with an intensive dairy industry [3]. Infection during pregnancy may result in abortion, depending on the stage of gestation when parasitaemia occurs, or may lead to the birth of a congenitally infected calf [4]. Treatment options are limited, with few chemotherapeutics available which may be problematic to use in meat or milk-producing livestock. Applying management and biosecurity measures such as those detailed in a management scheme recently launched by Defra in the UK (Herdsure) [5], may help to reduce infection levels in the herd; culling of seropositive animals has also been suggested as a method of control [6]. All these approaches can constitute a substantial cost for the farming industry.

There is accumulating evidence that cattle previously exposed to the parasite are less likely to abort than those undergoing a primary infection [7] suggesting the development of some form of protective immunity and the feasibility of a vaccination approach. To date only one commercial vaccine [8], based on an inactivated tachyzoite preparation adjuvated with Havlogen [9], has been registered in some countries. This vaccine demonstrated variable reduction in the number of abortions under field challenge condition in Costa Rica [10] and New Zealand [11]. However, it did not prevent foetal infection [12] and did not allow discrimination between vaccinated and naturally infected animals. Studies that have focussed on the evaluation of *N. caninum* tachyzoite proteins as vaccine candidates in mouse models have given ambiguous results, ranging from 70-90% protection using live attenuated tachyzoites [13] to very little or no protection

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with the SRS2 antigen and ISCOMs [14]. It appears that immunisation with live attenuated organisms is more effective than killed organisms, presumably as a reflection of more efficient antigen processing and presentation to T cells.

Immunological screening requires knowledge of the immune mechanisms responsible for protection, the so-called correlates of protection [15]. Cattle infected with *N. caninum* produce parasite-specific antibodies although their contribution to protective immunity is not clear [7,16]. There is mounting evidence that, as for other intracellular protozoan parasites, the most important correlate of protection for *N. caninum* is the establishment of a cell mediated immune response [7,17]. *In vitro* studies have shown that treatment of cultured cells with recombinant interferon gamma (IFN-γ), a cytokine produced by activated T-lymphocytes, significantly inhibits the intracellular multiplication of *N. caninum* [18]. A number of studies have also demonstrated that activated T-lymphocytes can recognise and respond to parasite-infected cells by producing inhibitory cytokines [19,20]. Staska et al. have shown a T-helper type 1 response in infected cattle involving $CD4^{+ve}$ cytotoxic T cells and IFN-γ production [21], whereas Boysen et al. showed that cytotoxic NK cells also play a role in the control of the disease through both cytotoxic and an IFN-γ mediated mechanisms [22]. Therefore, the development of vaccines directed against *N. caninum* should focus on selecting antigens that are capable of eliciting mainly a cell mediated immune response involving $CD4^{+ve}$ T cells and IFN-γ, in addition to a serological response.

The aim of this work was to identify *N. caninum* tachyzoite antigens that are recognised by the cell-mediated immune (CMI) response of experimentally infected animals. *Neospora caninum* water soluble antigens were initially separated by size exclusion HPLC and tested for their ability to induce proliferative responses in a NcWSA-specific bovine $CD4^{+ve}$ test system. A number of fractions which consistently induced significant proliferative responses were further investigated by tandem mass spectrometry allowing the identification of the proteins present. This type of approach demonstrated that is possible to use biologically relevant screening tools to select T-cell reactive fractions, thus facilitating the downstream analysis of relevant candidate vaccine antigens for *Neospora caninum*.

Materials and methods

Experimental design

Neospora caninum water-soluble antigen (NcWSA) was subdivided into smaller, less complex protein pools by size exclusion HPLC. Three identical aliquots of the same preparation of NcWSA were run in three consecutive size exclusion separations and tested *in vitro* for immune recognition by short-term NcWSA specific $CD4^{+ve}$ cell lines derived from cattle experimentally infected with *N. caninum*. Immuno-reactive fractions were subjected to SDS-PAGE and LC-ESI-MS/MS prior to downstream database mining and bioinformatic analysis to identify their respective protein compositions.

Parasites, inocula and immunisation schedules

Neospora caninum tachyzoites (NC1 isolate) [23] were maintained in Vero cells as previously described [18] and used to prepare infectious inocula as detailed below. Experimental live tachyzoite challenge confers protection against abortion [24] and has been employed in the past by us and other groups to characterise protective immune responses [25,26]. Calves (see below) were infected subcutaneously over the left pre-femoral lymph node with the live inoculum containing 1×10^8 tachyzoites per calf. A control inoculum containing an equivalent number of Vero cells as present in the parasite inocula was administered to control calves. All experimental animals employed in this study were reared, housed and handled in accordance to the UK Animals (Scientific Procedures) Act 1986; the experimental design was approved by the Moredun Ethical Review Committee. Five male calves, dehorned and castrated, aged two months and serologically negative for *N. caninum* antibodies by IFAT [27] and a commercial ELISA (NC Herdcheck, IDEXX Laboratories, Chalfont St Peter, UK) were randomly assigned to two groups. Three animals received the infectious inoculum whereas the two remaining animals received the control. Rectal temperatures were monitored from day 2 to day 14 post-infection (pi) and blood samples were collected up to one month post-infection for serological analysis. Seroconversion was confirmed by ELISA. Twelve months after the first inoculation the animals were boosted with a further similar dose of either live *N. caninum* tachyzoites or control Vero cell inoculum.

Lymphocyte Transformation Tests (LTT)

Initiation of a CMI response was confirmed by LTT (Lymphocyte Transformation Test) on Peripheral Blood Mononuclear Cells (PBMCs) isolated according to previously published protocols [28]. Briefly, PBMCs were resuspended in cell culture medium (CCM) (comprising IMDM [Gibco, Invitrogen, Paisley, UK], 10% heat inactivated foetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 5 μg/mL Amphotericin B [all from Sigma, Gillingham, UK]). PBMCs were cultured in 96-well plates (Nunc, Roskilde, Denmark) at concentration of 2×10^5/well and stimulated with 200 ng/well of antigen (NcWSA-see below) for 5 days in a humidified 5% CO_2 atmosphere at 37°C. Controls included cell culture media, Concanavalin A (ConA) (1 μg/well, Sigma, UK) and Vero cell lysate (200 ng/well). Cultures were pulsed with 1 μCi

of [^{3}H]-thymidine (GE HealthCare, Bucks, UK) for the final 18 h of incubation. Cells were harvested onto glass fibre filters (Wallac, Turku, Finland) and [^{3}H]-thymidine incorporation quantified using an automated scintillation counter (Perkin Elmer, Cambridge, UK) and expressed as counts per minute (CPM), with each test performed in quadruplicate. Stimulation indices (SI) were calculated by dividing the median value of the test by the median value of the media control.

Preparation of *N. caninum* Water-Soluble Antigen (NcWSA)

NcWSA was produced as follow: 1-2 × 10^9 tachyzoites, prepared accordingly to previously published methods [29] were washed three times in PBS (650 × *g* for 5 min) then stored at -20°C prior to antigen preparation. After thawing, tachyzoites were suspended in distilled water and disrupted by three cycles of freezing and thawing in liquid nitrogen followed by homogenisation using a Precellys tissue homogenizer (Precellys, Bertin Technologies, Tarnos, France). The homogenised suspension was centrifuged at 10 000 × *g* for 30 min at +4°C to recover the supernatant containing the *N. caninum* WSA. Protein concentration was assessed using the BCA reagent (Pierce Chemicals, Rockford IL, USA). NcWSA was then aliquotted and stored at +4°C prior to chromatographic fractionation, which was performed within 24 h of antigen preparation.

Size exclusion HPLC of NcWSA

Size exclusion chromatography was performed using a Beckman System Gold HPLC apparatus (Beckman Coulter, High Wycombe, UK) in combination with a Superose 12 gel filtration column (GE Healthcare) pre-equilibrated with PBS pH 6.8. Individual 200 mL injections of 0.45 mm-filtered NcWSA (935 mg total protein) were applied to the column and the proteins resolved isocratically in PBS pH 6.8 at a flow rate of 0.5 mL/min over a period of 60 min. Proteins eluting from the column were monitored by UV (280 nm) and chromatographic data was recorded and analysed using 32 Karat Gold ™ chromatograpy analysis software (Beckman Coulter). Fractions of 1.0 mL were collected and stored at +4°C in sealed low-protein-binding tubes (Eppendorf, Cambridge, UK) until required. Reproducibility of the fractionation was confirmed by overlaying the chromatograms of three consecutive separations as shown in results.

Protein quantification of individual antigen fractions was performed using the NanoOrange® protein quantification kit (Molecular Probes, Invitrogen, Paisley, UK) in accordance with the manufacturer's instruction and an automated fluorescence reader (CytoFluor, PerSeptive Biosystems, Framingham, MA, USA). Protein concentrations were then adjusted between fractions to ensure equal concentration of each fraction used in the T-cell lines proliferation assays, and fractions were stored at -20°C until required for the T cell assay.

Generation and characterization of bovine CD4^{+ve} T cell lines and antigen screening assay

Short-term antigen specific T cell lines were prepared from the infected animals as follows: for the first round of stimulation each well of a 96-well round bottom tissue culture plate (Nunc, Denmark) was seeded with 2 × 10^5 freshly isolated PBMCs resuspended in CCM and stimulated with 200 ng NcWSA. Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere for seven days then harvested, washed (300 × *g* for 10 min), resuspended in CCM supplemented with 10 U/mL of recombinant human IL-2 (rhu IL-2, Proleukin, Novartis, East Hanover NJ, USA) and seeded into fresh 96-well round bottom plates. Six days later an aliquot of cells was removed for phenotypical analysis and the remaining cells were cultured for a further 24 h prior to use in the antigen screening assay. The phenotypic composition of the short-term T cell lines was analysed using a panel of monoclonal antibodies (MoAbs) recognizing specific bovine leukocyte populations according to previously published methods [30]. Antibody binding was revealed with Alexa 488-conjugated anti-mouse IgG (Invitrogen, UK) (0.5 μg/mL final) and data acquired using a CyAn flow cytometer (CyAn, Dako-BeckmanCoulter, USA) equipped with a 488 nm argon-ion laser and analyzed using Summit software (Dako, Fort Collins, CO, USA). A minimum of 10 000 cells were acquired for each sample.

These short term lines prepared from the three infected animals were used to test each fraction generated from the three different HPLC runs (n = 9). Test wells for the T cell lines screening assays were set up in triplicate in 96 well round bottom tissue culture plates. Each well contained 5 × 10^4 T cells, 5 × 10^5 autologous antigen presenting cells (APC) (3000 rad γ-irradiated PBMCs; at a 1:10 ratio) and the different HPLC-separated fractions (1-25) at a final concentration of 10 ng/well. Negative controls comprised T cells or APCs with medium only as well as APCs plus T cells with only CCM or with Vero lysate. Positive controls included T cells, APCs or APCs plus T cells cultured with 500 ng/well ConA or unfractionated NcWSA with a final concentration comprised between 200 and of 10 ng/well. Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere and proliferation was quantified by ^{3}H-thymidine incorporation as described previously for the proliferation assay.

Shotgun proteomic analysis of selected reactive fractions

All protemics-based analysis were performed by the Moredun Proteomic Facility, (Moredun Research Institute, Penicuik, UK). A pool of each homologous fraction selected on the basis of the CD4^{+ve} T cell reactivity was

dialyzed overnight against HPLC-grade water (membrane cut-off 3 kDa), snap-frozen in liquid nitrogen then freeze-dried. Each pellet was resuspended in 25 μL of reducing SDS-PAGE sample buffer, heated at 95°C for 5 min, separated using a SDS-PAGE gel (4-12% Tris-glycine gradient, NuPage, Invitrogen, UK) and finally stained with Simply Blue Safe Stain ™ (Invitrogen, UK). The gel lanes were excised in their entirety then divided equally into slices of 2.5 mm deep to yield 25 gel slices. Each slice was then de-stained before processing using standard in-gel reduction, alkylation and trypsinolysis procedures [31]. The resulting peptides were analysed by Liquid Chromatography Electrospray Ionisation Tandem Mass Spetrometry (LC-ES-MS/MS) using a U3000 nano-flow UHPLC apparatus (Dionex, Camberley, UK) and amaZon high capacity ion trap mass spectrometer (Bruker, Coventry, UK). Parameters for tandem MS analysis were set as previously described [32]. Processed MS/MS data, in mascot generic format (mgf), was mined against a) the NCBInr database [33] using alveolata as taxonomical search and b) a cognate *Neospora caninum* genomic database (N.c. Liverpool strain) [34]. The presentation and interpretation of MS/MS data was performed in accordance with published guidelines [35]. A more detailed description of the Tandem Mass Spectrometry procedure can be found in the additional file 1.

Results

Clinical and immunological reactivity after challenge

Between 72 and 96 h after the initial *N. caninum* challenge, the infected animals showed pyrexia and swelling of the ipsilateral draining lymph nodes, whereas the negative control animals remained normal. In the infected animals seroconversion was demonstrated 14 days post infection (dpi) by ELISA (data not shown) and antigen specific CMI responses were detected one month after infection by LTT (SI values between 14 and 135). Control animals showed no seroconversion or antigen-specific responses (SI values between 2 and 3; data not shown). Twelve months after the initial challenge the infected animals were inoculated with a second live immunisation, using a similar dose and route as described in material and methods. Following the second immunisation, LTT reactivity was evident and serological reactivity was demonstrated using Western blot analysis (an example of Western blot reactivity is shown in an additional figure - additional file 2). The animals were employed to prepare $CD4^{+ve}$ T cell lines starting from three weeks after the second challenge.

HPLC fractionation of *N. caninum* water-soluble antigen

Corresponding fractions (1-25) from each of three identical size exclusion separations of NcWSA were combined to yield 25 pooled fractions. Chromatograms of each separation were superimposed to demonstrate consistency over the three replicate runs (Figure 1A). In addition, similarity between homologous fractions obtained from the three consecutive runs was confirmed by SDS-PAGE analysis on three representative fractions (Figure 1B). An example of SDS-PAGE analysis of the HPLC-separated fractions is shown in an additional figure (see additional file 3). These results demonstrate the robustness of the HPLC fractionation and show that this is independent from the size of the separated proteins.

Antigen fraction screening using CD4+ve T cell lines

Short term stimulation of immune PBMCs with NcWSA and IL-2 produced 2-week old cell lines that consistently comprised a majority of $CD3^{+ve}$ and $CD4^{+ve}$ T cells ($CD3^{+ve}$: average 97%, min 94.3% and max 98.5%; $CD4^{+ve}$ average 87%, min 77.2% and max 93.2%) with very low $CD8^{+ve}$ and γ/δ TCR^{+ve} T cells contamination (on average less than 5% for CD8 and 8% for γ/δ TCR^{+ve}). These cells were employed in the T cells assays for the antigen fraction screening test where we identified three fractions (fractions 3, 4 and 5) which consistently induced a cellular reactivity above all the other fractions tested (Figure 2). This reactivity corresponded, on the HPLC trace, to proteins collected between 14 and 20 min. from the start of the chromatographic separation. Fraction 3 showed the highest reactivity, closely followed by fraction 4, whereas fraction 5 was higher than all the remaining fractions but to a large extent lower than 3 and 4. Despite the presence of higher concentration of proteinaceous (as visualised by SDS-PAGE, additional file 3) and non-proteinaceus material (as deduced from the peaks in the HPLC trace) in other fractions, the remaining fractions were only marginally stimulatory to the T cell lines. In addition, fractions 17 to 25 consistently failed to induce reactivity above background level. On this basis, fractions 3, 4 and 5 were selected for further analysis.

Proteomic analysis of selective reactive fractions

Proteins contained within fractions 3, 4 and 5, identified as consistently reactive with the $CD4^{+ve}$ T cell lines, were separated by SDS-PAGE and catalogued by shotgun proteomic analysis using LC-ESI-MS/MS. For each individual fraction sample, a non-redundant list of identified proteins was prepared, and a master list was generated, which was termed as *N. caninum* reactive protein list (NCRP) which comprises a total of six unique *N. caninum* proteins (Table 1) as well as sixteen *T. gondii* homologues (Tables 2 and 3). Surface antigens, proteasome subunits, microneme and dense granule proteins as well as some putative uncharacterised proteins were identified. Three of the *N. caninum* proteins were present both in fractions 3 and 4 (SAG1, SRS2 and GRA2), two were found exclusively in fraction 3 (microneme protein Nc-MIC3 and GRA7) and

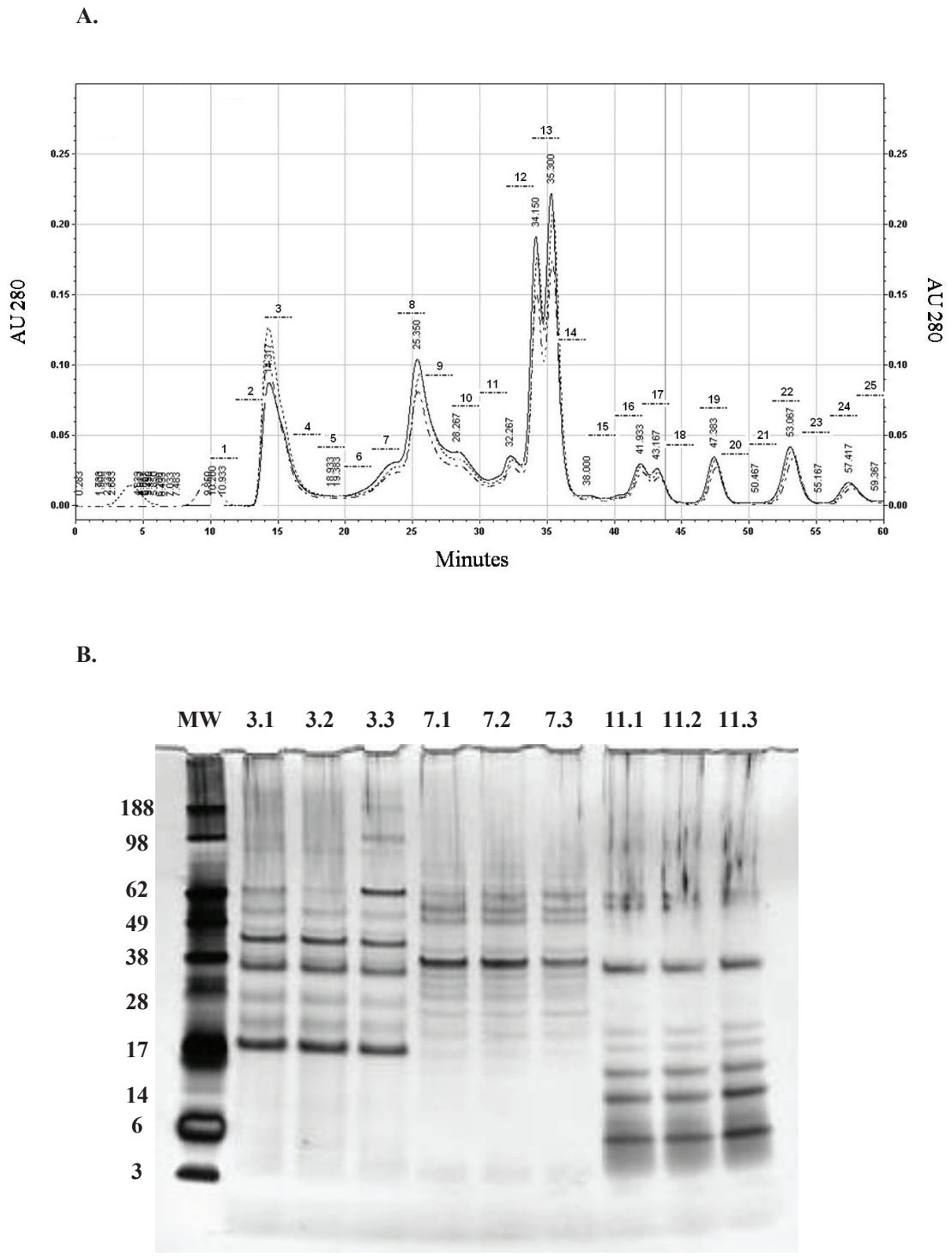

Figure 1 Size exclusion fractionation of *N. caninum* Water Soluble Antigen. (A). Three identical aliquots of NcWSA were divided into fractions comprising molecules of progressively lower mass by size exclusion HPLC. The superimposed absorbance profiles of the fractions generated in three successive runs are shown. Horizontal axis: time, vertical axis: 280 nm absorbance. Fraction collection started 10 min into the run, flow rate was 0.5 mL/min and a new fraction was collected every 2 min. The horizontal dotted lines indicate which portion of the NcWSA antigen corresponded to each collected fraction; bold numbers above the dotted lines represent the fraction number, vertical numbers represent retention time in minutes. **(B)**: Silver stained SDS PAGE gel (4-12% Bis-Tris gradient) of three representative fractions (3, 7 and 11) obtained in three separate runs (.1, .2 and .3) of size-exclusion chromatography. MW: molecular weight markers; 3.1: fraction 3 run 1; 3.2: fraction 3 run 2; 3.3: fraction 3 run 3; the numeration is equivalent for the further two fractions.

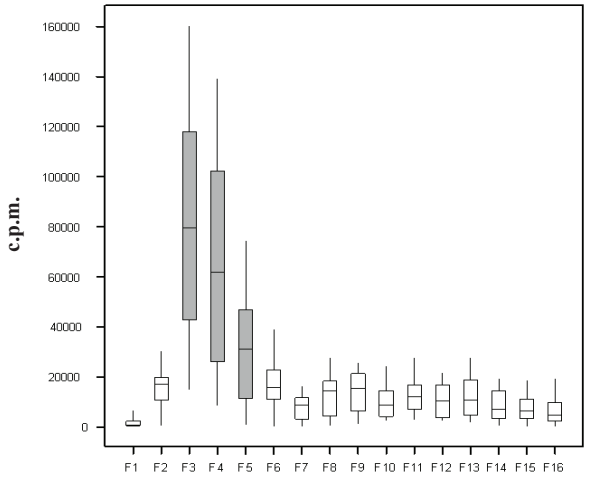

Figure 2 Proliferation of CD4^{+ve} T cell lines in response to the fractionated *N. caninum* water-soluble antigen. Average proliferative response of 9 different cell lines (three each from three animals) to the first 16 HPLC fractionated antigen preparations. Results are expressed as count per minute (cpm-vertical axis). F1-F16 represents the fraction number. Each box plot spans the interquartile range of cpm values, with the inside line indicating the median value. Whiskers extend as far as the minimum and maximum values. The fractions selected for further proteomic analysis are shadowed. Fractions 17 to 25 were not recognised or recognised at background level by the cell lines and are not represented in the figure.

only one was identified exclusively in fraction 4 (microneme protein NcMIC11). Homologues of *T. gondii* proteins identified comprised surface, ribosomal, proteasome, histone and rhoptry proteins, seven of which were identified in fraction 3, nine in fraction 4 and three in fraction 5. In some cases the same protein was indentified in contiguous fractions (Table 2) however, the majority of *T. gondii* homologues were present exclusively in a single fraction (Table 3).

Discussion

The primary objective of this work was to identify immunologically (cell mediated) relevant antigens of *N. caninum*, using a combination of proteomics-based and immunological approaches. As CD4^{+ve} T cells are important in disease protection [17] we expanded immune precursors from this population to screen tachyzoite antigens generated through size exclusion fractionation. Selected fractions were then analysed by LC-ESI-MS/MS to catalogue their respective protein profiles. This initial immuno-potency screening of the soluble fractionated antigens was deemed necessary because, whilst proteomic characterisation of an organism provides information on its composition and complexity, it does not always reflect the relative immunological importance of the molecules identified.

We initially demonstrate that size-exclusion fractionation using an aqueous mobile phase is highly reproducible and generates material that is free of detergents and salt concentrations that are incompatible with the *in vitro* CD4^{+ve} test, and for the same reason we opted to use a soluble preparation of the parasite as starting material. A selection based on Western blot reactivity (see additional file 2) would have suggested deeper interrogation of fractions 2 to 7, which correspond to the majority of the proteins present in the original water soluble antigen. However, a comparison of the serological reactivity profiles with the cellular reactivity suggested that only fractions 3, 4 and 5 would benefit from a further analysis. Shotgun proteomics analysis of the selected fractions led to the identification of six *N. caninum* reactive proteins as well as sixteen functional orthologues of *T. gondii* proteins.

Among the six proteins identified from mining the *N. caninum* database and common to both fractions three and four were the surface antigens SAG1 and SRS2 and the dense granule protein GRA2. SAG1 is a tachyzoite glycosylphosphatidylinositol (GPI)-anchored surface molecule [36] thought to be implicated in host cell attachment and invasion [37] and is serologically immunodominant [38]. Recombinant SAG1 immunisation has been attempted with inconsistent results in rodents using a vaccinia virus delivery vector [39], a cDNA prime-protein boost regime

Table 1 NCRP list of *N. caninum* proteins identified in reactive fractions

Fraction (s)	Accession	Gene name	Description	MOWSE	Peptides Matched	Example Peptides
3, 4	AAD25091	*SAG1*	Surface antigen SAG1*	1048	9	K.EIPLESLLPGANDSWWSGVDIK.T; K.SVSSPEVYCTVQVEAER.A
3, 4	AAX38598	*SRS2*	Surface protein SRS2**	760	9	K.LLSEDDGLIVCNESDGEDECEK.N; R.LRPITVNPENNGVTLICGPDGK.A
3, 4	AAG28489	*GRA2*	GRA2 protein	287	4	R.GTVNGQPVGSGYSGYPR.G; R.ESMAAPEDLPGER.Q
3	AAF19184	*MIC3*	Microneme protein Nc-MIC3	219	5	K.NPMCYPTCEEMGGK.D; K.DAECVEDLNAGGSVR.C
3	P90661	*DG1*	GRA7***	116	2	K.LAVPVVGALTSYLVADR.V; R.VLPELTSAEEEGTESIPGK.K
4	AAN16380	*NcMic11*	Microneme protein NcMIC11	137	2	K.STAVEIFK.Q; K.AAIVEGVKPMLPK.L

* also known as Nc-p29 or Nc-p36; ** also known as Nc-p35 or Nc-p43; *** also known as NCDG1 [39].

Table 2 Homologues of *T. gondii* proteins identified in more than one fraction

Fraction	Accession	Gene name	Description	MOWSE	Peptides Matched	Example Peptides
3, 4	XP_002369822		SRS domain containing proteins	821	16	K.IDLDPEDLHGHVYLPLVEQVDPMR.L; K.DLGQFGYVPPGDGRDPAGDEVQECK.Y
4, 5	XP_002365950		Glutamine synthetase, putative	168	4	K.IDPPPPADCDAAEVDSPLVR.S; R.TLVDAADLMMVYK.Y + 2 Oxidation (M)
4, 5	EEE20214		20S Proteasome subunit alpha	194	4	K.VEVEVGLIGNDSCGVFK.M; R.IAAVTETIGIAVAGLAADGR.Q

[40] or rSAG1 protein only [41]. SRS2 (or Nc-p43) [42] is localised on the surface of *N. caninum* of both bradyzoites and tachyzoites [43], is involved in the host cell invasion process [44] and its neutralisation inhibits parasite attachment and *in vitro* invasion of placental trophoblasts [45]. By homology with *T. gondii* we also identified a second SRS domain-containing protein in fractions 3 and 4, in addition to the *N. caninum* SRS2 protein. SRS-domain containing proteins are considered extremely immunogenic in *Toxoplasma* [46] as well as being present in a large number on the parasite surface, and are thought to facilitate the invasion of multiple host and cell types [47]. Therefore the identification of more than one of these proteins in our *N. caninum* reactive fractions is perhaps not surprising. NcSRS2 has been selected as candidate antigen for vaccination by a number of groups. Rodent challenge with *N. caninum* following vaccination with NcSRS2 demonstrated improved survival [41] reduced transplacental transmission [48] and the development of humoral and cellular immune responses to *N. caninum* tachyzoites [49]. In cattle, NcSRS2 peptide-specific T lymphocytes have been detected *ex vivo* in peripheral blood of infected animals [21]. Baszler et al. [50] also demonstrated the induction of a cell-mediated immune response similar to that induced by the live parasite in animals vaccinated with NcSRS2 in combination with Freund's adjuvant.

Table 3 Homologues of T. *gondii* proteins identified in only one fraction

Fraction	Accession	Gene name	Description	MOWSE	Peptides Matched	Example Peptides
3	EEE22451		Putative uncharacterized protein	837	7	K.LEVGETCTIEMLPQNSK.V; K.HKLEVGETCTIEMLPQNSK.V
3	EEE23072		Putative uncharacterized protein	194	4	R.ATVHPGDTVTMQCPGAISSNPADVSK.Y; R.LILDIEKSEEEVVR.T
3	EEE32684		Surface protein rhoptry protein	135	2	K.SQANQGSPLPPPRPNLLR.R; R.GLMSGVGWVK.R
3	EEE29336		Histone H4	110	2	R.ISGLIYEEIR.G; R.DNIQGITKPAIR.R
3	XP_002370897	*ROP 2*	Rhoptery protein 2	91	3	R.DSGDVILEELFK.R; K.GPSAIVFEATDR.E
3	EEE23774		Ribosomal protein S8	82	2	K.NSIVAIDATPFK.A; K.LDPLLEEQFNTGR.L
4	AAD38419		HSP 60	283	4	K.QVASTTNDIAGDGTTTATLLAR.A; K.TLTHELELVEGLK.F
4	XP_002369317		Proteasome subunit alpha (Type 2)	215	7	R.YNPDIELEDAIHTAILTLK.E; K.EGFEGAMNEHNIEIGVVGEDR.K + Oxidation (M)
4	EEE23454		Proteasome subunit alpha (Type 1)	182	3	K.ELSLDEIQALLDK.M; R.NFESFPGLSPEELELHAMK.A
4	XP_002366589		Proteasome subunit alpha (Type 4)	162	4	K.EDLDVDAALLLAAK.V; K.QEWKEDLDVDAALLLAAK.V
4	EEE19215		Proteasome subunit Beta [(Type 7)	100	2	K.GCAVVLGGVDFK.G; R.VSMAVSVLSQELFK.Y
4	EEE25357		Proteasome subunit alpha (Type 7)	129	2	K.DLVVLAVEK.K; R.LNTATAPSVDYIAK.F
5	EEE30125		Cytosol aminopeptidase putative	142	2	K.LTLFTDDVEAVNR.S R.VVTSFLETLLVELQPDLR.F

Proteins were identified in each fraction by blasting the results of the peptide analysis versus the *N. caninum* and *T. gondii* genomic.

Proteins were identified in each fraction by blasting the results of the peptide analysis versus the *N. caninum* and *T. gondii* genomic databases. Table 1 lists protein identified from the *N. caninum* database; Table 2 *T. gondii* homologues identified in more than one fraction and Table 3 *T. gondii* homologues present only in one of the three fractions. MOWSE scores (for Molecular Weight Search) indicate the likelihood of having correctly identified a specific protein from the molecular weight of the peptides created by its proteolytic digestion and measured with mass spectrometry. The dot (.) in the peptide sequence denotes trypsin cleavage sites.

In addition to surface expressed antigens, we also detected dense granule antigens such as NcGRA2 (p29) and NcGRA7. *Neospora caninum* GRA2 was originally identified by Ellis and collaborators [51] because of its significant amino acid sequence homology (50%) with the GRA2 antigen of *T. gondii*; similarly NcGRA7 (or dense granule protein 1) shows 42% identity with *T. gondii* GRA7 [42]. Dense granule antigens are specialised secretory organelles belonging to the parasitophorous vacuole synthesized at the time of infection and implicated in the cellular invasion process [52] as well as in nutrient acquisition [53]. NcGRA2 is another immunodominant antigen and is recognised by IgM from sera of *N. caninum*-infected cattle [54]. *E. coli* expressed NcGRA2 demonstrated immunogenicity but only partial reduction in foetal infection and pup mortality in a mouse model [55] and Ramamoorthy reported that vaccination of mice with recombinant NcGRA2 induced only partial protection against vertical transmission [56]. Two microneme proteins (Nc-MIC3 and NcMIC11) were also identified in the reactive fractions. Micronemes are secretory organelles which are discharged by exocytosis during the attachment to the host cell surface to facilitate cell invasion [57]. Despite their low molecular weight, microneme proteins could have been eluted in one of the early fractions as protein-complexes, since most of them have putative adhesive functions, are naturally secreted as multiprotein complexes, and immunoprecipitation experiments in *T. gondii* have confirmed that specific microneme proteins form a stable complex within the microneme [58]. In analogy with *T. gondii*, different microneme proteins such as NcMIC11, an ortholog of TgMIC11 [59], NcMIC1 [58] and NcMIC4 [60] have been identified in *N. caninum* but so far only one, Nc-MIC3, has been associated with immunological (serological) reactivity [36]. Use of microneme proteins in vaccination and challenge studies has given contradictory results in rodent models: vaccination with NcMIC4 increased mortality following challenge [61] whereas immunisation with NcMIC1 [62] or NcGRA7 [63] elicited only partial protection. Additional proteins from cellular cytoplasm (rhoptries, ribosomes, HP60), nucleus (hystones) as well as enzymes (proteasome complex, glutamine synthetase, cytosol aminopeptidase) and some additional molecules of unknown function were also found in the reactive fractions by homology with *T. gondii* proteome. The identification of TgROP2 homologue is also promising since immunisation with recombinant NcROP2 in a mouse model has been effective in reducing mortality and cerebral infection [64], in addition to reducing vertical transmission [65] when used in combination with two microneme antigens (NcMIC1 and NcMIC3).

Involvement of proteasome genes in the generation of a protective response to *N. caninum* in mice has been recently suggested by Ellis [66] and HSP60 has been identified as a serologically immunodominant protein [54]. *Neospora caninum* rhoptry antigens have also been identified as serologically immunodominant [67] while in *T. gondii*, some of the rhoptries proteins have been linked to increased virulence [68].

Cell-mediated antigen screening has in the past lead to the identification of parasite fractions capable of being recognised by memory T cells [69,70]. However, because the antigenic components of the parasite were not identified, these previous studies did not allow the selection of specific candidate antigens. Our approach, which combines cellular screening and proteomic characterisation, refines these previous investigations and show that it is possible to streamline the screening of biologically reactive fractions, narrowing the number of molecules of potential interest to a manageable size. Each identified protein can now be investigated to further select those capable of generating the correct *in vivo* immunological response.

Additional material

Additional file 1: Liquid Chromatography ElectroSpray Ionisation tandem Mass Spectrometry (LC - ESI-MS/MS) methodology and database mining information. extended methodological information on the execution of LC - ESI-MS/MS and database mining.

Additional file 2: Western blot reactivity of fractionated NcWSA after separation by size exclusion HPLC probed with a *N. caninum* positive serum. Western Blot image showing serological reactivity of fractionated *N. caninum* Water-Soluble Antigen, as well as short methodological information.

Additional file 3: SDS PAGE analysis of fractionated *N. caninum* Water-Soluble Antigen after separation by size exclusion. SDS-PAGE gel image showing proteic composition of HPLC fractionated *N. caninum* Water-Soluble Antigen, as well as short methodological information.

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Authors' contributions
MR, PB and EAI conceived the study and participated in its design and coordination; MR and PB carried out sample collection and cellular assays. ECF performed the serological (ELISA) analysis. NI performed the HPLC separation and the proteomic data collection whereas PB analysed the data. FK participated in the design and the organization of the study. MR, EAI and GE drafted the final version of the manuscript.
All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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6 Maternal and foetal immune responses of cattle following experimental challenge with *Neospora caninum* at day 70 of gestation

Paul M. Bartley, Stephen E. Wright, Stephen W. Maley, Colin N. Macaldowie, Mintu Nath, Clare M. Hamilton, Frank Katzer, David Buxton and Elisabeth A. Innes

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6.1 Manuscripts main hypotheses

The main hypotheses that were tested in this manuscript are

1 At what stage of early gestation are bovine foetuses capable of mounting an i*n utero* immune response against *Neospora caninum*?

2 What effect does the timing and location of the maternal immune response have on the outcome of pregnancy in dams experimentally challenged with *Neospora caninum* tachyzoites on day 70 of gestation?

3 Does the route of parasite administration have an effect on the clinical outcome of disease in dams experimentally challenged with *Neospora caninum* tachyzoites on day 70 of gestation?

The results from this experiment clearly demonstrate that the timing of a primary challenge with *Neospora caninum* during gestation is critical to foetal survival. A challenge during early pregnancy resulted in foetal deaths being recorded by day 14 post inoculation. The results also show that the route of inoculation plays a role in foetal survival, as 6/6 dams challenged intravenously (iv) all showed foetal death while a subcutaneous (sc) challenge only resulted in foetal deaths in 3/6 animals. This experiment also showed the importance of the maternal immune response in foetal survival. The dams carrying live foetuses demonstrated stronger cell mediated immune (CMI) responses than the dams carrying dead foetuses.

Though mitogenic responses were shown from foetal tissues from as early as day 84 of gestation indication the presence of lymphoid cells, no parasite specific CMI responses were recorded, demonstrating that the foetuses are still too immunologically immature during early gestation to mount a protective *in utero* immune response against the parasite.

One of the major limiting factors during this experiment was group size, with only 2 animals being studied for each group at each time point, more animals could have increased the power and significance of some of the observations made during this study. A more comprehensive examination of the role of the innate immunity (NK cell and TLR responses) particularly during the priming and initiation of the early maternal immune response would have been informative, especially when comparing the responses of dams from the sc challenged animals carrying live and dead foetuses.

6.2 Author contributions

PMB, SEW, SWM, CNM, CMH, DB and EAI were involved in all aspects of the experimental design and planning of the experiments. All experiments were approved by Moredun Research Institutes experimental ethics committee.

SEW, SWM and DB inoculated all of the cattle, collected routine blood samples for serological screening and were responsible for making clinical observations. PMB, SEW, SWM, CNM, CMH, DB and EAI were all involved in the post mortem examinations of both dams and foetuses and for collecting all of the tissue samples used for immunological and histological analysis.

PMB maintained the Vero cells and *Neospora* tachyzoites in tissue culture. PMB and EAI enumerated the cells and tachyzoites using a Neubauer haemocytometer and prepared all of the inocula (Vero cells and tachyzoites) used during the experiment. PMB, CH and EAI performed lymphocyte stimulation assays on PBMC and lymph node and spleen samples from both dams and foetses, through the incorporation of ^{3}H thymidine and collected the cell stimulation assay supernatant samples. PMB analysed the supernatant samples from dams and foetuses by ELISA for the presence of the cytokines IL-4, IL-10, IL-12 and IFN-γ. PMB collated all of the data and prepared all of the graphs and tables presented in the manuscript.

SEW separated serum from clotted foetal blood samples from and performed all serological testing (IgM and IgG) by IFAT, while SWM, CNM and DB performed all histological and immunohistological analysis.

MN performed all of the statistical analysis on the proliferation and IFN-γ cytokine ELISA data from the PBMC and lymph node and spleen samples from both dams and foetuses.

PMB, SEW and EAI drafted the original manuscript with contributions from all other authors.

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Maternal and foetal immune responses of cattle following an experimental challenge with *Neospora caninum* at day 70 of gestation

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Abstract

The immune responses of pregnant cattle and their foetuses were examined following inoculation on day 70 of gestation either intravenously (iv) (group 1) or subcutaneously (sc) (group 2) with live NC1 strain tachyzoites or with Vero cells (control) (group 3). Peripheral blood mononuclear cell (PBMC) responses to *Neospora* antigen and foetal viability were assessed throughout the experiment. Two animals from each group were sacrificed at 14, 28, 42 and 56 days post inoculation (pi). At post mortem, maternal lymph nodes, spleen and PBMC and when possible foetal spleen, thymus and PBMC samples were collected for analysis. Inoculation with NC1 (iv and sc) lead to foetal deaths in all group 1 dams (6/6) and in 3/6 group 2 dams from day 28pi; statistically significant ($p \leq 0.05$) increases in cell-mediated immune (CMI) responses including antigen-specific cell proliferation and IFN-γ production as well as increased levels of IL-4, IL-10 and IL-12 were observed in challenged dams compared to the group 3 animals. Lymph node samples from the group 2 animals carrying live foetuses showed greater levels of cellular proliferation as well as significantly ($p \leq 0.05$) higher levels of IFN-γ compared to the dams in group 2 carrying dead foetuses. Foetal spleen, thymus and PBMC samples demonstrated cellular proliferation as well as IFN-γ, IL-4, IL-10 and IL-12 production following mitogenic stimulation with Con A from day 14pi (day 84 gestation) onwards. This study shows that the generation of robust peripheral and local maternal CMI responses (lymphoproliferation, IFN-γ) may inhibit the vertical transmission of the parasite.

Keywords: *Neospora caninum*, Cattle, Early gestation, Maternal – foetal cellular immune

Introduction

The protozoan parasite *Neospora caninum* is a major cause of abortion and reproductive failure in cattle worldwide. The most common route of infection with *N. caninum* appears to be the transplacental (vertical) transmission of the parasite from mother to foetus; this may result in abortion or the birth of clinically normal but persistently infected offspring [1,2]. Horizontal transmission of the parasite may occur in intermediate hosts through the ingestion of oocysts (shed by a definitive host i.e. dog) in contaminated feed and water [3], potentially leading to point source outbreaks (abortion storms) of neosporosis. Previous studies in cattle have shown that *N. caninum* infections can be maintained over several generations through vertical transmission of the parasite [1,4], Moen *et al.* (1998) demonstrated that as a result of a primary infection, cattle were 3–7 times more likely to abort than uninfected animals [5]. However, animals that have aborted due to neosporosis are less likely to abort due to the parasite during subsequent pregnancies, compared to cows undergoing *N. caninum* infection during their first pregnancy [6], suggesting that a certain level of protective immunity builds following infection. Experimental data by Innes *et al.*, (2001) [7] demonstrated that exposure of cattle to *Neospora* prior to pregnancy protected against the vertical transmission of the parasite following an experimental challenge with *N. caninum* during pregnancy. Other factors influencing the outcome of *N. caninum* infections in pregnant cattle include; the quantity and duration of the parasitaemia [8], the

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parasite strain (as some have been shown to be more virulent than others, in cattle) [9], the immune status of the dam and the gestational age of the foetus at the time of infection [7,8]. Experimental infections of pregnant cattle have shown that foetal death may occur when dams were challenged with *N. caninum* tachyzoites at day 70 of gestation [10,11], while a challenge administered around mid gestation resulted in the vertical transmission of the parasite, but no foetal death [12,13]. These observations would suggest that the timing of a parasitaemia during pregnancy is critical in the clinical outcome and will likely be influenced by both the maternal and foetal immune responses to the parasite. Work carried out by Williams *et al*. (2000); Collantes-Fernandez *et al*., (2006) and Rosbottom *et al*., (2007) [10,14,15] would support this conclusion.

The intracellular nature of *N. caninum* suggests that a cell-mediated immune (CMI) response is likely to be important to protect the host [12]. Increasing experimental data from pregnant cattle has confirmed this [7,15-18]. Work by Bartley *et al*., (2004) [13] demonstrated a strong CMI response in dams and foetuses challenged with *N. caninum* on day 140 of gestation. Although, no foetal deaths were recorded, vertical transmission of the parasite occurred and the maternal and foetal immune responses appeared to contribute to the resolution of infection. Numerous other studies have illustrated the importance of a pro-inflammatory T-helper (Th)-1 type response, interferon-γ (IFN-γ) in particular has been shown to be crucial in controlling infection both *in vivo* [15,16,19] and *in vitro* [20-22]. The timing and location of these pro-inflammatory immune responses has also been shown to be critical to the clinical outcome of a *Neospora* infection in cattle. Work by Maley *et al*., (2006) [23] demonstrated in cattle experimentally challenged with *Neospora* on day 70 of gestation; that the infiltration of large numbers of immune cells and increased levels of expression of IFN-γ mRNA in the placenta lead to foetal death and abortion.

In this study, we compared the maternal and foetal immune responses in cattle inoculated either intravenously (iv) or subcutaneous (sc), with live *N. caninum* (NC1 strain) tachyzoites at day 70 of gestation. A serial examination of the maternal and foetal immune responses was conducted looking at *Neospora* specific cell proliferation and cytokine production in PBMC and lymph node samples following experimental challenge.

Materials and methods

Animals, inoculum and experimental design

Twenty four pregnant Holstein-Friesian cattle aged 1.3 to 4 years and seronegative for *N. caninum*, *Toxoplasma gondii*, bovine viral diarrhoea virus, infectious bovine rhinotracheitis and *Leptospira hardjo* were assigned into three groups. Pregnancy and foetal viability was confirmed in all experimental animals by ultrasound scanning 36 days after insemination. On day 70 of gestation, group 1 dams (n = 8) received an intravenous (iv) inoculation in the right jugular vein of 5×10^8 live *N. caninum* (NC1 isolate) tachyzoites. Group 2 dams (n = 8) received a subcutaneous (sc) inoculation of 5×10^8 live *N. caninum* (NC1 isolate) tachyzoites over the left pre-femoral lymph node. Group 3 (n = 8), the control animals each received an iv inoculation of 5×10^6 Vero cells. This dose of Vero cells was used, as it was the equivalent number of cells present in the parasite inocula. Blood was collected by weekly jugular venipuncture throughout the experiment for immunological analysis. Two animals from each group were sacrificed at days 14, 28, 42 and 56 post inoculation (pi). At post mortem samples of left pre-femoral lymph node (LPF), right pre-femoral lymph node (RPF), left uterine lymph node (LUL), right uterine lymph node (RUL), mesenteric lymph node (MLN), retropharyngeal lymph node (RLN), spleen and peripheral blood mononuclear cells (PBMC) were collected from each dam; When possible spleen, thymus and PBMC samples were collected from the foetuses.

Preparation of cells for immunological assays

Single cell suspensions of PBMC were prepared as previously described [7]. Samples from lymph nodes collected at post mortem were prepared as previously described [13]. In brief, excess fat was trimmed from the tissues, which were then cut into small pieces and placed in 10 ml wash buffer (Hank's balanced salt solution (HBSS) supplemented with 2% foetal bovine serum (FBS) (Labtech International, Ringmer, UK) 100 IU/ml penicillin and 50μg/ml streptomycin) (Northumbria Biologicals, Cramlington, UK), placed in a stomacher bag (Seward Medical, Northampton, UK) and homogenised for 10 seconds. The resultant cell suspension was decanted into a sterile universal through sterile lens tissue to remove clumps of cells, washed twice by repeated centrifugation at 260 × *g*, counted using a Neubauer haemocytometer and resuspended at a final concentration of 2×10^6 cells/ml in cell culture media (CCM) (Iscoves modified Dulbecco's media (IMDM) (Gibco, Paisley, UK) supplemented with 10% FBS, 100 IU/ml penicillin and 50μg/ml streptomycin).

Cell proliferation assays

Single cell suspensions of both PBMC and lymph node tissues were treated as previously described [13]. In brief, equal volumes (100 μl) of cells (2×10^6/ml) and antigen were added in quadruplicate to 96-well round bottom plates (Nunc, Roskilde, Denmark). Water-soluble *N. caninum* tachyzoite antigen (NCA) [7] was used at a final protein concentration of 1μg/ml, the T-cell mitogen

concanavalin A (Con A) was used as a positive control at a final concentration of 5μg/ml, CCM alone was used as a negative control to determine the background level of proliferation. A Vero cell lysate antigen at 1 μg/ml was used as a control antigen. The cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere for 5 days. The cultures were pulsed with 18.5 kBq ^{3}H Thymidine/well (Amersham Biosciences, Little Chalfont, UK) for the final 18 hours, before being harvested onto glass-fibre filters (Canberra Packard, Meriden, CT, USA) and the cell-associated radioactivity was quantified using a MATRIX 96^{TM} gas proportional counter.

Cytokine responses

Duplicate cell proliferation assays were prepared to those described above. Cell free supernatants were collected after 4 days incubation, to measure the levels of secreted cytokines (interferon-gamma (IFN-γ), interleukin-4 (IL-4), IL-10 and IL-12). The supernatant samples were stored at −20°C prior to analysis.

IFN-γ

Levels of IFN-γ production were quantified using a commercially available enzyme linked immunosorbent assay (ELISA) kit (CSL Veterinary, Parkville, Australia). A standard curve was generated using doubling dilutions of known quantities (ng/ml) of recombinant bovine IFN-γ (rBoIFN-γ) (Pfizer Animal Health, Parkville, Australia). Mean optical density (OD) values were plotted against ng/ml rBoIFN-γ and a standard regression curve was fitted to the data. Experimental samples were extrapolated against the standard regression curve to determine the levels of IFN-γ in the test samples.

IL-10

Levels of bovine IL-10 were quantified using an ELISA method as previously described [24]. In brief, 96-well ELISA plates (Greiner, Stonehouse, UK) were coated with 50μl (4μg/ml) per well with a primary anti-bovine IL-10 capture antibody and incubated at room temperature overnight. The plates were washed x 5 using phosphate buffered saline (PBS) supplemented with 0.05% Tween 20 (PBS-T) between each step, with the exception of the final TMB – H_2SO_4 stage. The plates were blocked at room temperature for 1 hour with PBS-T supplemented with 3% bovine serum albumin (BSA). Samples and standards (50μl each) were added and incubated at room temperature for 1 hour. Plates were then coated with (1μg/ml) secondary biotinylated anti IL-10 antibody (Diluted in PBS-T 1% BSA) (50μl per well) and incubated at room temperature for 1 hour. Streptavidin-horseradish peroxidase (HRP) (Dako Cytomation, Glostrup, Denmark) diluted 1:500 in PBS-T 1%BSA (50μl/well) was added and incubated at room temperature for 45 minutes. Colour was developed by the addition of TMB (3,3′,5,5′-tetramethylbenzidine) substrate (Insight Biotech. Ltd., Wembley, UK) (100μl/well) and incubated in the dark for 10–15 minutes. Reactions were stopped by adding 50μl/well 1 M H_2SO_4. The plates were read at 450/650 nm using a MRX II plate reader (Dynex, East Grinstead, UK). Doubling dilutions of known quantities of recombinant bovine IL-10 (rBoIL-10) were used to generate a standard regression curve against which the test sample concentrations were extrapolated.

IL-12

Interleukin-12 (IL-12) was quantified using the same method described above for the detection of IL-10, with the following changes. A primary anti IL-12 capture antibody (4μg/ml) was used along with a secondary biotinylated anti IL-12 antibody (8μg/ml). Known quantities of recombinant ovine IL-12 (rOvIL-12) were used as standards and standard regression curve was fitted to the data [25].

IL-4

Interleukin-4 (IL-4) was quantified using the same method described above for the detection of IL-10 and IL-12, with the following changes. A primary anti IL-4 capture antibody (6μg/ml) was used along with a secondary biotinylated anti IL-4 antibody (2μg/ml). Known quantities of recombinant bovine IL-4 (rBoIL-4) were used as standards and a standard regression curve was fitted to the data [26].

All primary and secondary antibodies used for the capture and detection of IL-4, IL-10 and IL-12 were purchased from AbD Serotec, (Oxford, UK). All rBoIL-4, rBoIL-10 and rOvIL-12 cytokines (Moredun Research Institute, Edinburgh, UK)

Foetal serology

At post mortem examination blood was drawn from the foetuses (when available) into non-heparinised evacuated tubes (Vacutainer, Becton Dickinson, Oxford,UK) and allowed to clot before centrifugation at 2000 x ***g*** for 15 minutes, the serum was removed and stored at −20°C prior to being tested for IgM and IgG to *N. caninum* by an indirect fluorescent antibody test (IFAT), as previously described [27]; an IFAT titre of ≥1:64 was considered positive.

Statistical analysis

The maternal cell proliferation data (PBMC, lymph nodes and spleen) and IFN-γ ELISA data were analysed with a linear mixed model, using a first-order autoregressive model to specify the temporal covariance structure. Both the proliferation and IFN-γ ELISA data were normalised by logarithmic transformation (base 10) prior to the analysis. The linear mixed model included the animal

effect as a random effect and the treatment, day and the interaction effect of treatment and day as fixed effects. Parameters of the linear mixed models were estimated using the REML method and *p*-values were estimated using the modified *F*-statistic. If the *F*-statistic was statistically significant ($p \leq 0.05$), two-sided probabilities for each treatment comparison were obtained; these probabilities were then adjusted using a False Discovery Rate approach [28]. This value, denoted in this paper as p_f, therefore summarises the strength of evidence for there being a real difference in a way analogous to a standard *p*-value.

The foetal proliferation and IFN-γ data (PBMC and spleen) were analysed using non-parametric Kruskal-Wallis one way analysis of variance (ANOVA) using treatment as a grouping factor. No data was available for group 1 for the foetal thymus, (proliferation and IFN-γ ELISA) hence a two sample non-parametric Mann–Whitney test was conducted. All statistical analyses were carried out using GenStat 13th Edition software (VSN International, Hemel Hempstead, UK)

Results

Clinical observations, pathology and foetal mortality

Foetal viability following experimental challenge is shown in Additional file 1. In group 1 (iv) viable foetuses were only seen on day 14 pi, both foetuses were found dead on day 28 pi and no foetuses were found on days 42 and 56 pi. In group 2 (sc) two viable foetuses were found on day 14 pi and one live and one dead foetus on days 28, 42 and 56 pi. In the group 3 (control) animals, two live foetuses were found at each time point. The maternal serology and histopathology data from this experiment is described in Macaldowie *et al*., (2004) *Neospora caninum* parasites were only demonstrated in placental and foetal tissues from challenged animals carrying dead foetuses and they were associated with lesions [11].

Maternal PBMC

Cell proliferation

The results from the PBMC proliferation assays from group 1 (iv) demonstrated statistically significantly higher mean levels of antigen-specific proliferation on days 14 ($p_f = 0.020$), 21 ($p_f = 0.004$), 42 ($p_f = 0.043$) and 56 pi ($p_f < 0.001$) compared to group 3 (control). The mean cell proliferation results from group 2 (sc) were found to be statistically significantly higher than group 3 on days 7 ($p_f = 0.004$), 14 ($p_f = 0.011$), 21 ($p_f < 0.001$), 42 ($p_f < 0.001$), 49 ($p_f = 0.020$) and 56 pi ($p_f < 0.001$). A comparison of the data from group 1 and group 2 showed that the mean antigen-specific proliferation in group 2 was higher than group 1 on day 7 pi ($p_f = 0.003$). At subsequent time points, the mean level of antigen specific PBMC proliferation of group 2 animals were higher in comparison to group 1 animals, though these differences were not statistically significant. (Additional file 2).

Cytokine responses

The levels of antigen-specific cytokine production was determined using the cell-free supernatants from maternal PBMC, lymph nodes and spleen samples following stimulation with NCA for 4 days

IFN-γ

The $\log_{10}$ transformed antigen specific-IFN-γ data from PBMC cultured with NCA (Additional file 3). Group 1 (iv) showed a rise in IFN-γ from days 7 to 21 pi and reached a peak on day 28 pi, then declined to baseline levels on day 56 pi. Though group 1 maintained an elevated level of IFN-γ compared to the group 3 (control) animals, there was no evidence of a difference between the mean IFN- γ levels of the two groups ($p_f = 0.107$). In group 2 (sc), levels of IFN-γ were consistently higher than other groups at all time points and reached a peak on day 21 pi. The mean level of IFN-γ from group 2 was statistically significantly different from that of group 3 ($p_f = 0.005$), though there was no evidence of a difference between the mean IFN-γ levels of group 1 and 2 ($p_f = 0.118$).

IL-4

In group 1 (iv) demonstrable antigen-specific IL-4 was observed in PBMC samples of one dam on day 14 pi (1.543U/ml), IL-4 production was seen in PBMC from both group 1 dams on day 28 pi (8.37U/ml and 2.54U/ml); while on days 42 and 56 pi PBMC samples from group 1 were below the detection level of the ELISA. Detectable levels of IL-4 were only seen in the PBMC from one of the group 2 (sc) animals on day 28 pi (carrying a dead foetus), these levels were much lower (0.518U/ml) than those seen in group 1, though no statistical differences were observed between groups 1 and 2. The levels of IL-4 in the group 3 (control) PBMC were either below the detection threshold of the ELISA at 0.114U/ml or only just detectable (data not shown).

IL-10

Demonstrable antigen-specific IL-10 was observed in PBMC from one group 2 (sc) animal carrying a dead foetus on 42 pi (0.286U/ml). All PBMC samples from groups 1 (iv) and 3 (control) were below the detection threshold (0.17U/ml) of the ELISA at all time points (data not shown).

IL-12

Levels of antigen-specific IL-12 were demonstrable in most PBMC samples collected from all three groups on days 14, 28 and 42 pi. Group 2 (sc) animals demonstrated higher

levels of IL-12 than the other two groups. On day 28 pi the group 2 dam carrying the dead foetus demonstrated 39.1U/ml while, 94.3U/ml was recorded from the dam carrying the live foetus. By day 56 pi levels of IL-12 were either below the detection threshold (0.3U/ml) or only just detectable in all three groups (data not shown).

Maternal lymph nodes and spleen

Cell proliferation

The $\log_{10}$ transformed antigen specific proliferation in maternal lymph nodes and spleen samples following stimulation with NCA. On day 14 pi, samples of LPF from group 2 (sc) had significantly ($p_f = 0.010$) higher mean proliferation than group 3 (control). While mean proliferation from spleen samples from groups 1 (iv) and group 2 were significantly higher ($p = 0.05$ and $p = 0.047$ respectively) than group 3 (Additional file 4).

On day 28 pi, samples of LPF from group 1 and 2 had significantly higher mean proliferation than group 3 ($p_f = 0.006$ and $p_f = 0.025$ respectively), while samples of RLN and RPF from group 1 showed higher values than groups 2 and 3 ($p_f = 0.023$ and $p = 0.05$ respectively) and ($p = 0.033$ and $p = 0.015$ respectively) (Additional file 4).

On day 42 pi, samples of LPF from group 2 and 3 had significantly higher mean proliferation than the group 1 ($p_f = 0.005$ and $p_f = 0.031$ respectively) (Additional file 4).

On day 56 pi, group 2 demonstrated statistically significantly higher mean proliferation than group 3 from RPF ($p_f = 0.051$), LPF ($p_f < 0.001$), RLN ($p_f = 0.023$) and RUL ($p = 0.021$) and MLN ($p = 0.007$) samples. Group 2 also demonstrated significantly higher mean proliferation than group 1 for LPF samples ($p_f < 0.001$), while group 1 had significantly higher mean proliferation than group 3 for RLN ($p_f = 0.023$) and RUL ($p = 0.021$) samples (Additional file 4).

Cytokine responses

IFN-γ

Following the stimulation of cells from spleen and lymph nodes with NCA for 4 days; group 1 (iv) and group 2 (sc) both showed detectable levels of antigen specific-IFN-γ (ng/ml) in all samples tested from day 14 pi onwards, while group 3 (control) produced almost undetectable levels of IFN-γ at each of the time points tested (Additional file 5). The mean levels of IFN-γ from group 1 was statistically significantly higher than group 3 for RPF ($p_f = 0.001$), RUL ($p_f < 0.001$), LUL ($p_f = 0.013$), RLN ($p_f = 0.003$) and spleen ($p_f = 0.001$). Similarly, the mean levels of IFN-γ from group 2 were statistically significantly higher than group 3 for RPF ($p_f = 0.004$), LPF ($p_f = 0.005$), RUL ($p_f = 0.005$), RLN ($p_f = 0.009$) and spleen ($p_f = 0.004$). There was no evidence in the present data that the mean IFN-γ values of group 1 and 2 were different for any of the lymph node or spleen.

IL-4

Group 1 (iv) showed demonstrable antigen-specific IL-4 from both dams for all spleen and lymph node samples (except for LPF and RPF – only one dam responded) on day 28 pi and in LUL of one dam on 42 pi. All samples tested from group 2 (sc) and group 3 (control) were either below the detection threshold of the ELISA at 0.114U/ml or only just detectable and hence no statistical analysis was undertaken (data not shown).

IL-10

Group 1 (iv) was the only group with demonstrable antigen-specific IL-10, this was seen in LUL (2.61U/ml) and RLN (1.27U/ml) samples on day 42 pi, All samples tested from group 2 (sc) and group 3 (control) were below the detection threshold of the ELISA and hence no statistical analysis was undertaken (data not shown).

IL-12

Group 1 (iv) had demonstrable levels of antigen-specific IL-12 in RPF, LUL, RUL, MLN, RLN and spleen on days 14, 28 and 42 pi, with the highest levels being observed on day 42pi (126.75U/ml, 210.55U/ml, 98.00U/ml, 114.71U/ml, 307.9U/ml and 137.09U/ml respectively). By day 56 pi the levels of IL-12 had dropped below the detection threshold of the ELISA (0.3U/ml). Group 2 (sc) demonstrated levels of IL-12 below or barely above the threshold in most samples on days 14 and 28 pi. Levels of IL-12 peaked on day 42 pi, in the RLN and spleen, (23.64U/ml and 33.23U/ml respectively); by day 56 pi levels of IL-12 had dropped below threshold. Group 3 (control) showed low but detectable levels of antigen specific IL-12 from samples at all time points. Due to paucity of data, no statistical analysis was undertaken (data not shown).

Foetal PBMC, thymus and spleen

Cell proliferation

Samples collected from the foetuses for immunological analysis are shown in Table 1. No *Neospora* antigen-specific cell proliferation was demonstrated from any of the samples from any of the foetuses tested.

From day 14 pi (day 84 of gestation) PBMC samples showed proliferation following stimulation with Con A (Figure 1). On day 28 pi (day 98 of gestation) mitogenic proliferation was demonstrated in the spleen and thymus of foetuses from both group 2 (sc) and group 3 (control). On day 42 pi (day 112 of gestation) proliferative responses to Con A were continued to be seen in PBMC, thymus and spleen samples from the foetuses from both group 2 and group 3. On day 56 pi (day 126 of gestation) PBMC, spleen and thymus all showed proliferation following Con A stimulation (Figure 1). When the responses of the foetal tissues were compared, no

Table 1 Foetal samples collected at post mortem examination

	Number of foetal samples collected/Total number of foetuses								
	Group 1 (iv)			Group 2 (sc)			Group 3 (Control)		
Day pi	PBMC	Thymus	Spleen	PBMC	Thymus	Spleen	PBMC	Thymus	Spleen
14	2/2	0/2	2/2	2/2	0/2	2/2	2/2	0/2	2/2
28	0/2‡	0/2‡	0/2‡	0/2	1/2‡	1/2‡	0/2	2/2	2/2
42	0/2†	0/2†	0/2†	1/2‡	1/2‡	1/2‡	2/2	2/2	2/2
56	0/2†	0/2†	0/2†	1/2‡	1/2‡	1/2‡	0/2	2/2	2/2

‡ – Foetus found dead *in utero* (No Samples able to be collected).
† – No foetus found (due to resorption or abortion).

statistical differences were observed between the groups in the levels of mitogenic proliferation.

Cytokine responses

Following stimulation with NCA, levels of antigen-specific IFN-γ IL-4, IL-10 and IL-12 were below detectable limits in all the foetal samples tested.

IFN-γ

The levels of IFN-γ following Con A stimulation on Day 14 pi (day 84 of gestation) were below detectable limits for all the samples tested. On days 28, 42 and 56 pi (days 98, 112 and 126 of gestation respectively) PBMC, spleen and thymus samples produced demonstrable quantities of IFN-γ, following stimulation with Con A. There were no significant differences observed in the levels of IFN-γ produced in Con A stimulated cells from any of the groups, at any of the time points tested (data not shown).

IL-4

Following Con A stimulation, on day 14 pi all samples tested from all three groups were below the detection threshold of the ELISA. On day 28 pi (day 98 of gestation), production of IL-4 was demonstrated in one group 3 (control) foetal thymus sample (0.117U/ml). On day 42 pi (day 112 of gestation) one group 3 foetal thymus (0.178U/ml) and both group 3 spleen (0.221U/ml, 0.214U/ml) samples produced demonstrable IL-4. On day 56 pi (day 126 of gestation), IL-4 was produced by the thymus samples of all three foetuses (group 2 and 3) and in the PBMC samples from both group 3 foetuses (data not shown).

IL-10

Following Con A stimulation, on day 14 pi all samples tested from all three groups were below the detection threshold of the ELISA. On day 28 pi (day 98 of gestation), IL-10 was produced by one group 3 (control) foetal spleen sample (0.587U/ml). On day 42 pi (day 112 of gestation) IL-10 was produced by group 2 (sc) and group 3 thymus samples (0.201U/ml and 0.194U/ml respectively); and in thymus, (0.359U/ml) spleen (0.947U/ml) and PBMC (1.681U/ml) samples from both group 2 and group 3 on day 56 pi (day 126 of gestation) (data not shown).

IL-12

Following Con A stimulation, IL-12 was expressed in foetal spleen and PBMC samples from all three groups from as early as day 14 pi (day 84 of gestation). Production of IL-12 continued to be demonstrated in all tissues tested from both group 2 (sc) and group 3 (control); peaking in the thymus (8.66U/ml) and spleen (12.17U/ml) on day 28 pi (day 98 of gestation) and in PBMC (12.92U/ml) on day 56 pi (day 126 of gestation) (data not shown).

Foetal serology

The results from the foetal anti-*Neospora* IgG IFAT show that on day 28 pi (day 98 of gestation) a serum sample collected from one of the foetuses from group 1 (iv) which was found dead *in utero*, gave a positive IFAT result with a titre of 1:128. On days 42 and 56 pi (days 112 and 126 of gestation respectively) no foetuses were present for sample collection. In groups 2 (sc) and 3 (control), serum samples were collected from live foetuses, these all tested negative for IgG IFAT (titre ≤1:64). All the samples tested from all the groups were negative (titre ≤1:64) for anti-*Neospora* IgM (data not shown).

Comparison of maternal immune responses in group 2 (sc) dams carrying live or dead foetuses

The immune responses of the dams in group 2 (sc) carrying live or dead foetuses were compared; The dam carrying a live foetus demonstrated considerably higher levels of cellular proliferation in LUL and RUL on days 28, 42 and 56 pi compared to the dam carrying the dead foetus; while on days 42 and 56 pi higher levels of proliferation were also seen in the PBMC of the dams carrying the live foetus compared to the dams carrying the dead foetuses. Though there were no significant differences seen in the levels of maternal proliferation, when the $\log_{10}$ data were compared using a linear mixed model, the dams carrying the live foetuses generally demonstrated higher levels of cell proliferation in PBMC and

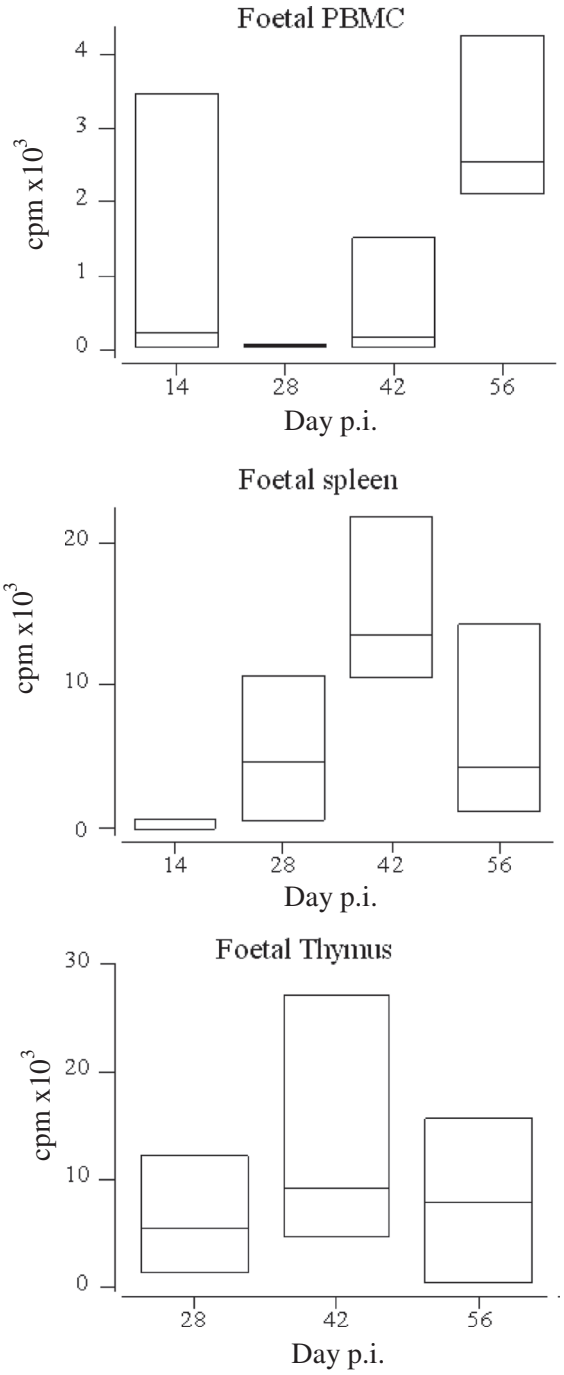

Figure 1 Proliferative responses of foetal PBMC, spleen and thymus samples following stimulation with Con A for 5 days. Samples of PBMC, spleen and thymus were collected at post mortem examination when available. Samples were stimulated with Con A for 5 days (37°C in a humidified 5% CO_2 atmosphere), with 18.5 kBq ^{3}H Thymidine/well being added for the final 18 hours, before being harvested onto glass-fibre filters. The box plots illustrate the combined counts per minute (cpm) $\times 10^3$ values from the foetal samples from all three group s at each time point. The boxes represent the upper and lower values obtained for each sample, the horizontal line = the median value.

lymph nodes stimulated with NCA, compared to the dams carrying the dead foetuses (data not shown). Comparisons were also made in the levels of antigen specific-cytokine responses (IFN-γ, IL-4, IL-10 and IL-12) in PBMC and lymph nodes of dams carrying live foetuses compared to the dams carrying dead foetuses in group 2. On day 28 pi, greater levels of antigen specific-IFN-γ were seen in samples from the LPF, LUL, RUL and RPF from dams carrying live foetuses compared to the dams carrying dead foetuses (data not shown). On days 42 (Figure 2A) and 56 pi, (Figure 2B) dams carrying live foetuses demonstrated increased levels of IFN-γ in all samples except RLN (on day 56 pi) compared to the dams carrying dead foetuses. When IFN-γ data from days 28, 42 and 56 pi were combined and analysed using a linear mixed model, the IFN-γ responses in the RPF and spleen of dams carrying live foetuses were statistically significantly higher than in dams carrying dead foetuses ($p = 0.008$ and $p = 0.005$ respectively). Dams carrying live foetuses also showed higher levels of antigen specific-IL-12 on day 28 pi (RPF, LUL, RUL, spleen and PBMC). On day 42 pi, the dam with the live foetus had higher levels of antigen specific-IL-12 in RLN (23.64U/ml), spleen (33.27U/ml) and PBMC (94.30U/ml) compared to the dam carrying a dead foetus (1.23U/ml, 13.9U/ml, and 39.11U/ml respectively). None of the dams carrying live foetuses had demonstrable antigen specific-IL-4 at any time point, while one dam carrying a dead foetus on day 42 pi showed antigen-specific IL-4 production in LUL, RUL, MLN, spleen and PBMC. No differences were seen in levels of antigen-specific IL-10 production between the dams carrying live and dead foetuses at any of the time points tested.

Discussion

This paper examines the maternal and foetal cell-mediated immune (CMI) responses of pregnant cattle experimentally inoculated with live *N. caninum* tachyzoites on day 70 of gestation (for a full description of the clinical observations and pathological findings see Macaldowie *et al*., (2004) and Maley *et al*., (2006) respectively) [11,23]. The route of inoculation of *N. caninum* tachyzoites played an important role in the outcome of infection, with 100% of group 1 (iv) animals having dead, reabsorbed or aborted foetuses from day 28 pi, compared to only 50% of the group 2 (sc) animals. The dams in group 2 (sc) proved to be very interesting, allowing us to compare the CMI responses in dams carrying live compared to dead foetuses following an identical challenge. Although the number of animals in the experiment was limited these results will aid our understanding of a protective maternal immune response, which is critical for foetal survival, due to the immaturity of the foetal immune response at this stage of gestation.

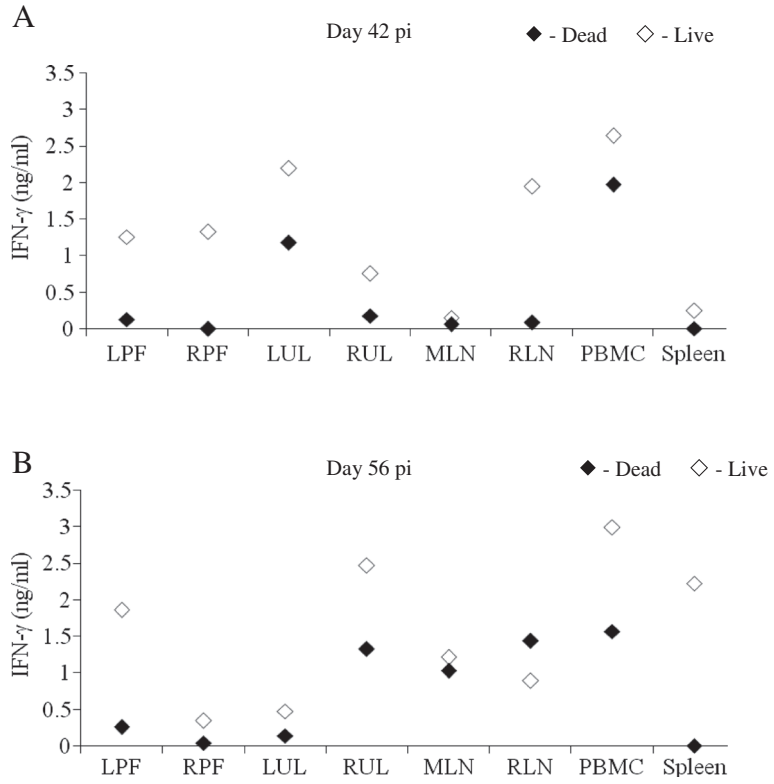

Figure 2 Comparison of IFN-γ production by group 2 (sc) dams carrying live or dead foetuses. Maternal lymph node, spleen and PBMC samples were collected at post mortem examination. Following stimulation with NCA for 4 days (37°C in a humidified 5% CO_2 atmosphere), ELISA were performed to determine the concentration of IFN-γ produced. The data is expressed as ng/ml. –◇– Live, –◆– Dead.

Mitogenic responses were detected in the foetuses from day 84 of gestation (day 14 pi) and anti-*Neospora* IgG was demonstrable from day 98 of gestation (day 28 pi).

Maternal immune response

The CMI responses observed in the dams in this study provides further evidence that cell-mediated immunity and in particular IFN-γ is important in protection against the rapidly multiplying intracellular tachyzoite stage of the parasite. Our data would suggest that a strong Th1 type cell-mediated immune response in both the peripheral and uterine lymph nodes as well as in PBMC, in particular in the group 2 (sc) dams carrying live foetuses, appears to have been sufficient to prevent the transplacental transmission of the parasite. When the immune responses of the dams in group 2 (sc) carrying live and dead foetuses were compared, it was seen that dams carrying live foetuses tended to exhibit stronger antigen-specific lymphocyte proliferation, significantly higher levels of IFN-γ as well as increased IL-12 production in samples of PBMC and lymph nodes, compared to dams in the same group (sc) carrying dead foetuses. Work carried out by Williams *et al*., (2000) [10] demonstrated that infection of pregnant cattle at 10 weeks gestation with *N. caninum* was accompanied by high levels of IFN-γ and lymphoproliferative responses indicating a profound Th1 helper T-cell like response in PBMC. Rosbottom *et al*., (2007) [15] observed proliferation of $CD4^+$ T-cells and the expression of IFN-γ and IL-4 in PBMC stimulated with *N. caninum* antigen in pregnant cattle challenged on day 70 of gestation.

However, when a strong immunological response occurs in the placenta during early gestation, this can be detrimental to the pregnancy. Davies *et al*., (2004) [29] demonstrated that the activation of the maternal mucosal immunity within the uterus during early placentome development, may lead to immune mediated abortion. During our study, the group 2 (sc) animals carrying dead foetuses (examined by Maley *et al*. (2006) [23]) demonstrated infiltration of large numbers of T lymphocytes, γδ T-cells, NK cells and IFN-γ mRNA, leading to extensive immune mediated damage (necrosis) of the placenta, compared to the dams carrying the live foetuses who showed mild or no infiltration of the immune cells, no demonstrable IFN-γ mRNA and consequently little or no immune mediated placental damage. Similar observations were made by Rosbottom *et al*., (2008) [30] who found in pregnant cattle, following a challenge with *N.*

caninum in early gestation, foetal death was associated with extensive placental necrosis, which corresponded to $CD4^+$ T-cell and macrophage infiltration and increased expression of IFN-γ.

Route of infection

Our data shows that the route of inoculation plays a critical role in the outcome of infection, with iv inoculation ($5x10^8$ live tachyzoites) leading to a greater incidence of foetal mortality than a sc inoculation with the same dose of parasites. This may be due in part to the haemotogenous nature of the iv inoculation, allowing the rapid transportation of the parasites to the placenta, before an effective maternal CMI response can be initiated to limit the spread of the tachyzoites. Comparing the immunological responses generated by the different routes of inoculation, group 2 animals (sc) demonstrated stronger proliferative responses in PBMC (by day 7 pi) and many of the lymph nodes (by day 14 pi) than the group 1 (iv) animals. Superior priming of the immune responses in the group 2 (sc) animals appears to have prevented the transplacental transmission of the parasite in 50% of cases. The animals in group 2 (sc) carrying live foetuses may have initiated an innate immune response more rapidly than the group 1 (iv) animals. This initiation is likely through the interaction of pathogen associated molecular patterns (PAMP) with toll like receptors (TLR) on the surface of antigen presenting cells (APC); this interaction induces a signalling pathway that will in turn lead to the activation of APC's and the production of pro-inflammatory cytokines, as well as leading to the maturation and migration of dendritic cells (DC) to local draining lymph nodes [31]. This APC migration may have been more rapid in the group 2 (sc) animals, as they were inoculated over a lymph node (left pre femoral lymph node). To date there is little experimental evidence demonstrating a role for the innate immune response in bovine neosporosis; work by Boysen *et al*., (2006) [20] and Klevar *et al*., (2007) [32] demonstrated a role for Natural killer (NK) cells during early infection in calves, in producing IFN-γ, and observed NK cytotoxicity in *N. caninum* infected fibroblast cells. Work in mice by Dion *et al*., (2011) [33] demonstrated that both DC and macrophages secrete IL-12 following exposure to viable *N. caninum* parasites, and that interactions between T lymphocytes and DC were involved in inducing IFN-γ production by T lymphocytes. Evidence from the closely related parasite *Toxoplasma gondii* has demonstrated an important role for innate mechanisms in a protective immune response including TLR [34-36]. Our experiment demonstrates through serial analysis; differences in the PBMC as well as lymph node responses of animals experimentally challenged by two different routes of inoculation (iv and sc). These differences appear to have been sufficient to protect against the vertical transmission of the parasite in some sc inoculated animals, compared to the iv challenged animals where vertical transmission and foetal death occurred in all animals from day 28 pi onwards.

Foetal immunology

As ruminants have a syndesmochorial placentation, which does not usually allow the transplacental passage of maternal immune factors including immunoglobulins and cytokines [37], any immune responses detected in the foetus are likely to be due to a response to an active infection *in utero*. Our study showed that foetuses at day 98 of gestation (day 28 pi) are capable of mounting a humoral (IgG) immune response against *Neospora*. This finding would agree with work by Senogles *et al*., (1979) [38] who demonstrated that at 3 months (approx 90 days) gestation bovine foetal PBMC contained B cells that labelled positively for the presence of IgG. By day 98 gestation, components of a cell-mediated immune response are also starting to develop. Our study demonstrated the presence of lymphoproliferative responses as well as the production of IFN-γ, IL-4, IL-10, and IL-12 being observed in foetal spleen, thymus and PBMC samples following mitogenic (Con A) stimulation. Schultz *et al*., (1973) [39] demonstrated in cattle the development of follicles of lymphoid thymus from as early as day 42 of gestation, while the spleen appears to develop from day 55 of gestation; while, Senogles *et al*., (1979) [38] demonstrated the presence of T-cells in the bovine foetal thymus, spleen and PBMC from 3 months of gestation. Work carried out by Hein *et al*., (1988) [40] showed that by day 120 of gestation bovine foetal lymphocytes were well developed and capable of mitogenic stimulation as well as the production of IL-2. Our work would suggest that this development occurs by around day 100 of gestation. The lack of any *Neospora*-specific CMI or humoral responses in the live foetuses from the group 2 (sc) dams was due to the fact that the foetuses were naïve to *Neospora*, due to no vertical transmission having occurred. The lack of histopathological lesions or demonstrable *Neospora* antigen in the placentomes or foetal tissues [11] indicates that vertical transmission had not occurred in the group 2 (sc) dams carrying live foetuses.

Other studies looking at the development of foetal immunology in cattle experimentally infected with *Neospora* have demonstrated that foetuses from infected dams at day 131 of gestation are capable of producing both Th1 and Th2 type cytokines [13,16]. Bartley *et al*. (2004) [9] showed that the foetuses from dams inoculated with *N. caninum* at mid gestation (day 140) are capable of mounting a specific cell-mediated immune response from 14 days post inoculation (day 154 gestation). While Andrianarivo *et al*. (2001) [17] demonstrated

strong CMI responses 9 weeks post challenge in bovine foetuses (days 219–231 of gestation).

Timing of infection

We demonstrate that experimental inoculation of cattle at day 70 of gestation can lead to foetal mortality between 14–28 days post inoculation (pi) (days 84–98 of gestation). Studies by Barr *et al*., (1994), Williams *et al*., (2000), Collantes-Fernandez *et al*. (2006) and Rosbottom *et al*., (2007, 2008) [14,30,41,42] have demonstrated that experimental infections with *N. caninum* in the first trimester of pregnancy leads to increased foetal mortality and higher parasite burdens and greater dissemination of parasites in foetal tissues than infections at mid or late pregnancy in cattle. Though Rojo-Montejo *et al*., 2009b observed no demonstrable foetopathy in cattle challenged on day 70 of gestation with the *Neospora* isolate Nc Spain 1 H [43]. While, Williams *et al*., (2000), Maley *et al*. (2003), Rosbottom *et al*., (2007) and Almeria *et al*., (2011) [10,12,15,44] demonstrated that a primary challenge in naïve pregnant heifers around mid gestation led to the transplacental transmission of the parasite but no foetal death. The results from these studies demonstrate that the timing of a primary infection during pregnancy is critical to the survival of the foetus. As gestation progresses the severity of a foetal infection decreases but the chances of congenital infection increases.

Conclusions

Our study demonstrates the development of maternal and foetal immune responses in lymph nodes and PBMC following challenge with live *N. caninum* tachyzoites. The innate immune response in the group 2 (sc) dams carrying live foetuses appears to have lead to superior priming of a cell mediated immune response, which inhibited the vertical transmission of the parasite, compared to the group 2 dams carrying dead foetuses. The route of inoculation of *N. caninum* tachyzoites has an impact on the clinical outcome of pregnancy in cattle, with an iv route of inoculation resulting in greater incidence of foetal mortality and less efficient immune priming compared to a sc inoculation of the same number of parasites. Foetuses from day 84 of gestation are capable of producing cellular responses to the mitogen Con A; these include lymphocyte proliferation as well as cytokine (IFN-γ, IL-4, IL-10, and IL-12) production. Our data has also shown that by day 98 of gestation foetuses are able to mount a humoral (IgG) response to *Neospora*. However should vertical transmission of the parasite occur, the foetuses are still too immunologically immature to mount a protective immune response, resulting in foetal mortality.

Additional files

Additional file 1 Foetal viability results following either iv (group 1) or sc (group 2) inoculation with live NC1 strain tachyzoites.

Additional file 2 Log_{10} transformed maternal PBMC proliferation data following stimulation with NCA for 5 days.

Addition file 3 Log_{10} Transformed IFN-γ results from maternal PBMC following stimulation with NCA for 4 days.

Additional file 4 Log_{10} transformed proliferative responses from maternal lymph nodes and spleen samples following stimulation with NCA for 5 days.

Additional file 5 Levels of antigen specific-IFN-γ (ng/ml) produced by maternal lymph node and spleen samples following stimulation for 4 days with NCA.

Abbreviations

iv: Intravenously; sc: Subcutaneously; PBMC: Peripheral blood mononuclear cell; CMI: Cell-mediated immune; IFN-γ: Interferon-gamma; IL-4: Interleukin-4; IL-10: Interleukin-10; IL-12: Interleukin-12; Con A: Concanavalin A; Th1: T-helper 1; Th2: T-helper 2; LPF: Left pre-femoral lymph node; RPF: Right pre-femoral lymph node; LUL: Left uterine lymph node; RUL: Right uterine lymph node; MLN: Mesenteric lymph node; RLN: Retropharyngeal lymph node; FBS: Foetal bovine serum; CCM: Cell culture media; IMDM: Iscoves modified Dulbecco's media; NCA: *N. caninum* tachyzoite antigen; CO_2: Carbon dioxide; ELISA: Enzyme linked immunosorbent assay; PBS: Phosphate buffered saline; BSA: Bovine serum albumin; TMB: 3,3',5,5'-tetramethylbenzidine; H_2SO_4: Sulphuric acid; HRP: Horseradish peroxidase; IFAT: Indirect fluorescent antibody test; IgM: Immunoglobulin M; IgG: Immunoglobulin G; ANOVA: Analysis of variance.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

PMB, SEW, SWM, CNM, DB and EAI made substantial contributions to the conception and design. PMB, CMH and EAI were involved in the acquisition of data. PMB and MN were involved in the analysis of the data. PMB, MN, FK and EAI have been involved in the drafting and critical review of the manuscript. All authors read and approved the final manuscript

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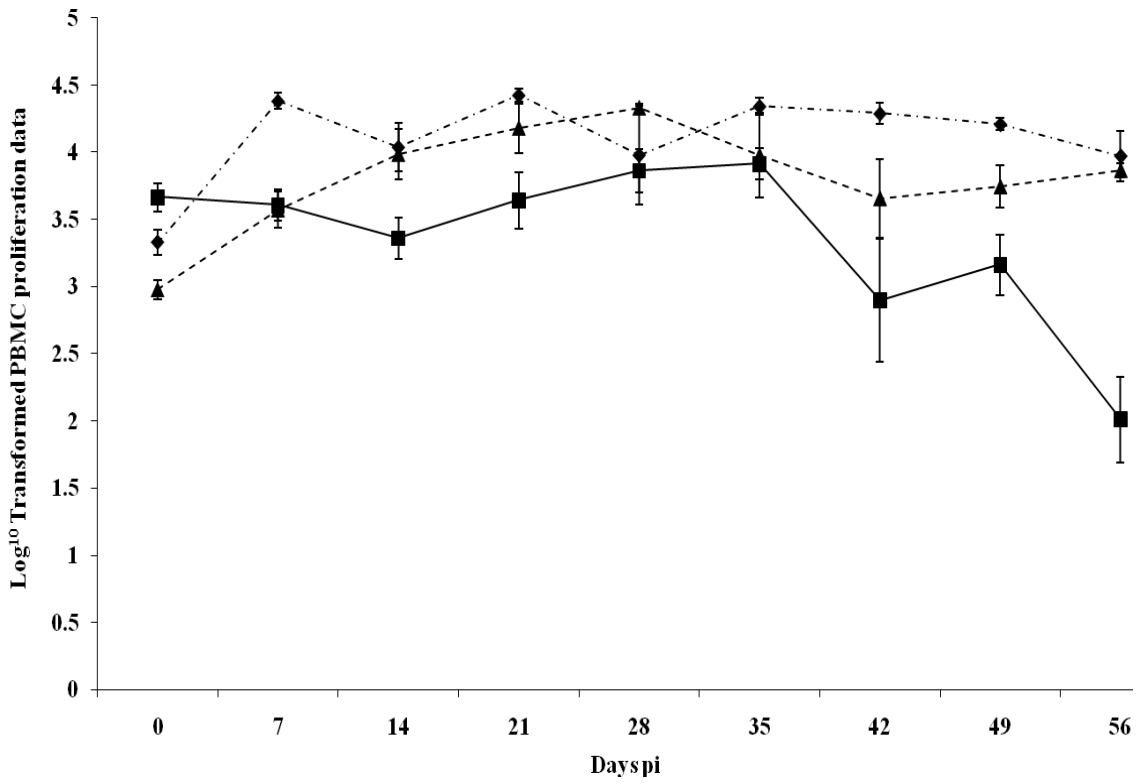
Additional file 1: Foetal viability results following either iv (group 1) or sc (group 2) inoculation with live NC1 strain tachyzoites

		Days post inoculation			
		Number of live viable foetuses / Total number of foetuses			
Group	Inoculation	14	28	42	56
1 (n=8)	$5x10^8$ NC1 (iv)	2 / 2	0 / 2‡	0 / 2†	0 / 2†
2 (n=8)	$5x10^8$ NC1 (sc)	2 / 2	1 / 2‡	1 / 2‡	1 / 2‡
3 (n=8)	$5x10^6$ Vero cells	2 / 2	2 / 2	2 / 2	2 / 2

‡ - Foetuses found dead *in utero*

† - No foetus found (due to resorption or abortion)

Additional File 2. Log_{10} transformed maternal PBMC proliferation data following stimulation with NCA for 5 days

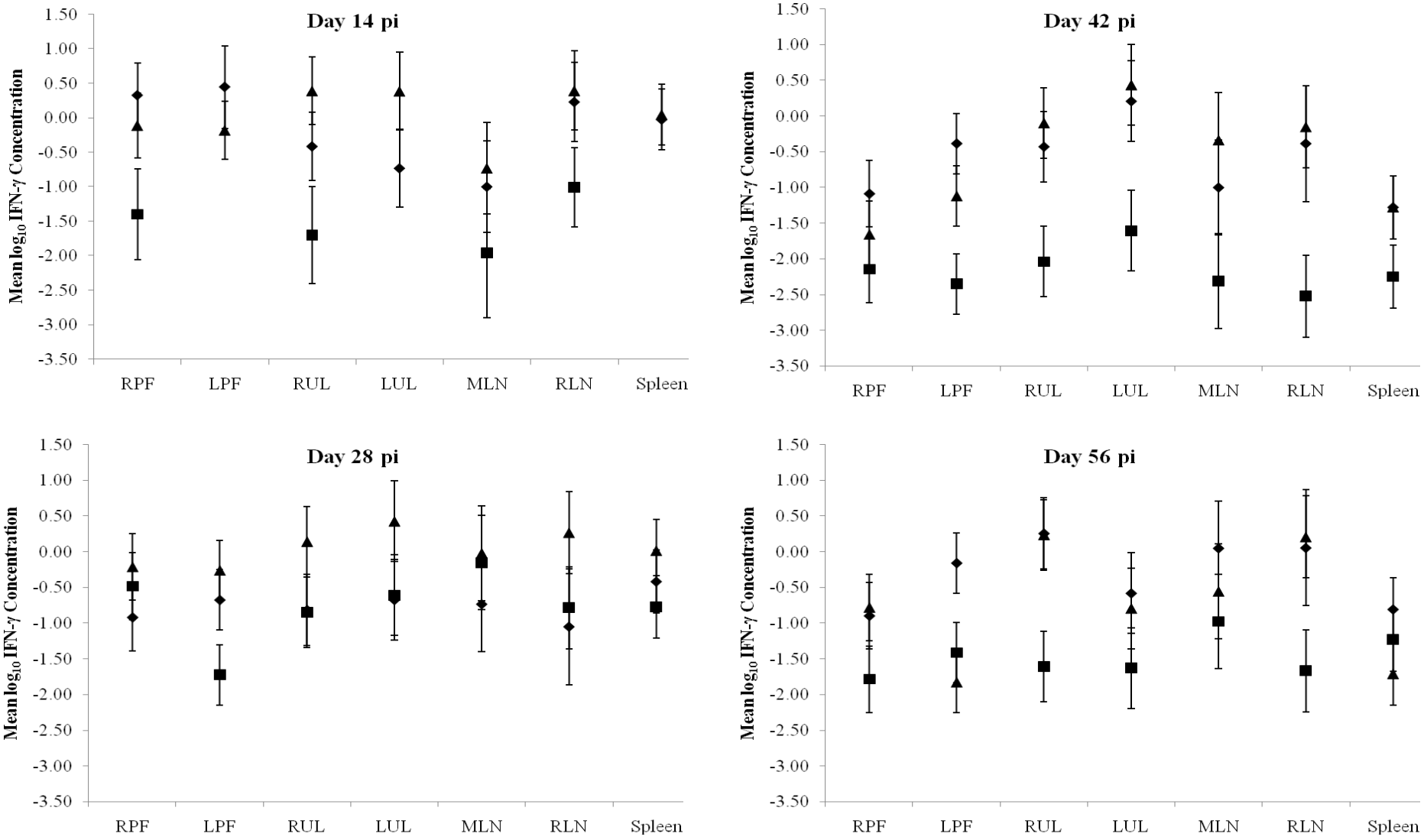


Blood was collected by weekly venapuncture throughout the experiment and processed to determine levels of *Neospora*-specific proliferation. The processed cells were stimulated with NCA for 5 days (37^{o}C in a humidified 5% CO_2 atmosphere), with 18.5kBq ^{3}H Thymidine / well being added for the final 18 hours, before being harvested onto glass-fibre filters. The data was then log^{10} transformed before analysis using a linear mixed model

-▲- Group 1 (iv), -♦- Group 2 (sc), -■- Group 3 (Control)

Error Bars Error bars (± standard error of the mean (S.E.M.))

Additional file 3. Mean Log_{10} antigen-specific IFN-γ concentrations from maternal lymph node and spleen samples following stimulation for 4 days with NCA.

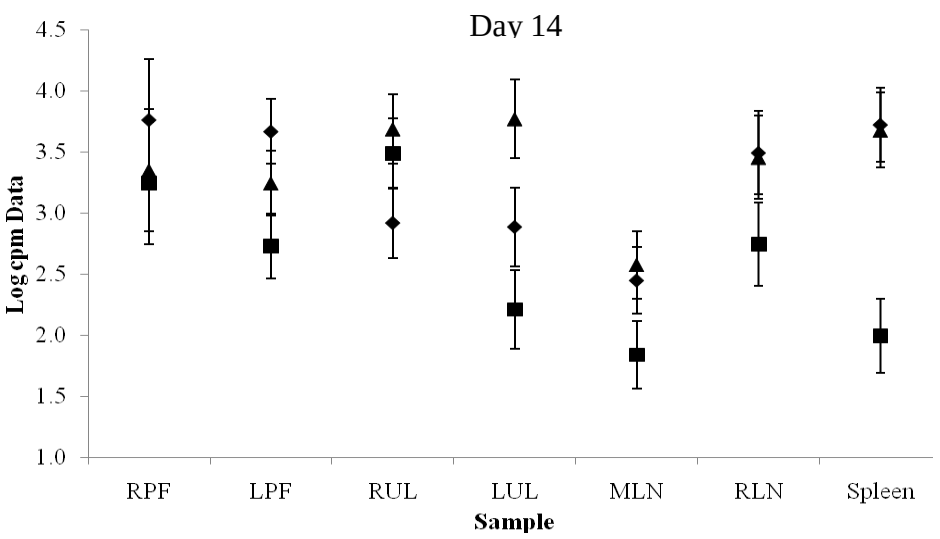


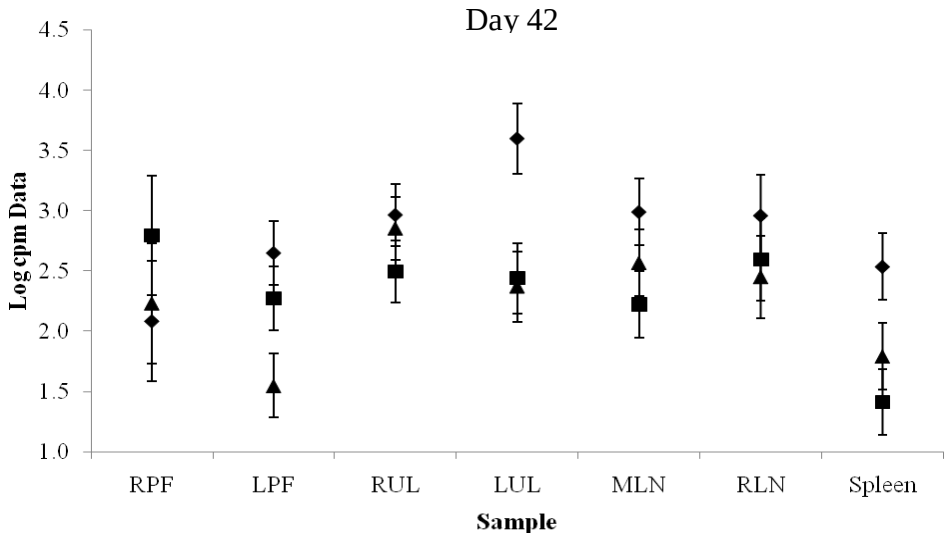
Mean Log_{10} antigen-specific IFN-γ concentrations from maternal lymph node and spleen samples following stimulation for 4 days with NCA

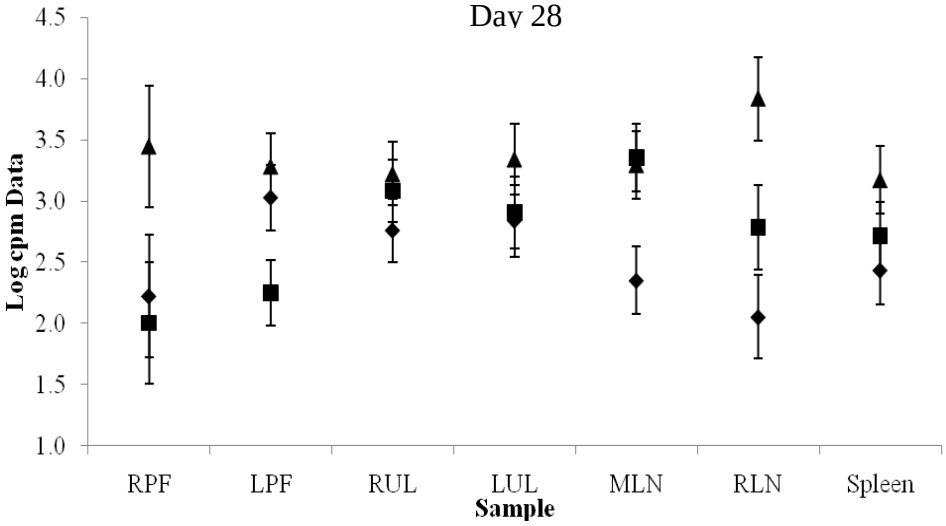
Lymph node and spleen tissue samples were collected at post mortem examination and processed to determine levels of *Neospora*-specific proliferation. The processed cells were stimulated with NCA for 4 days ($37^{o}C$ in a humidified 5% CO_2 atmosphere); ELISA were performed to determine the concentration of IFN-γ produced.

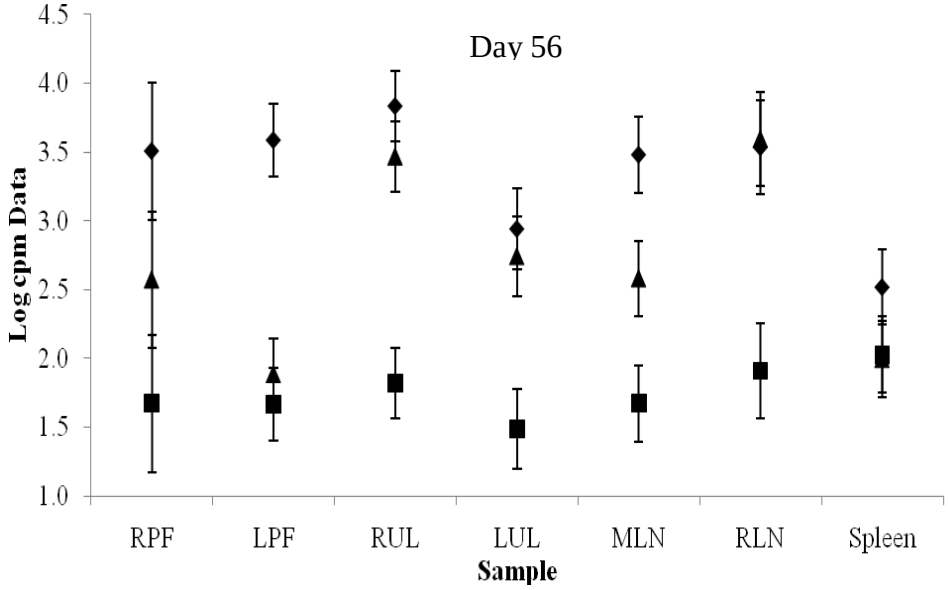
-▲- Group 1 (iv), -♦- Group 2 (sc), -■- Group 3 (Control). Error bars (± standard error of the mean (S.E.M.))

Additional File 4. Log_{10} transformed proliferative responses from maternal lymph nodes and spleen samples following stimulation with NCA for 5 days.







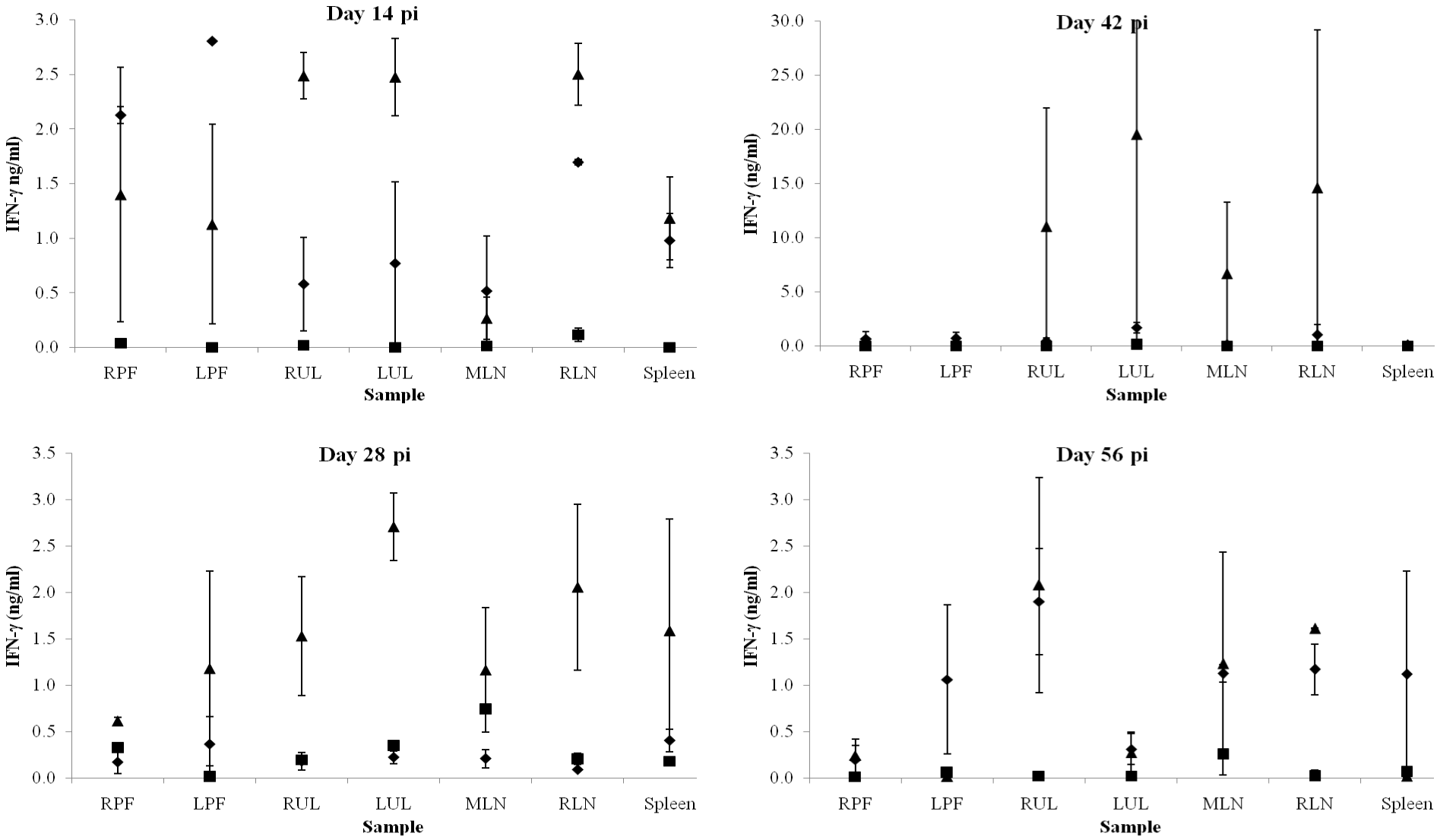


Log_{10} transformed proliferative responses from maternal lymph nodes and spleen samples following stimulation with NCA for 5 days

Lymph node and spleen tissue samples were collected at post mortem examination and processed to determine levels of *Neospora*-specific proliferation. The processed cells were stimulated with NCA for 5 days ($37^{o}C$ in a humidified 5% CO_2 atmosphere), with 18.5kBq ^{3}H Thymidine / well being added for the final 18 hours, before being harvested onto glass-fibre filters. The data was then log_{10} transformed before analysis using a linear mixed model.

-▲- Group 1 (iv), -♦- Group 2 (sc), -■- Group 3 (Control) Error Bars (±S. E.)

Additional file 5. Levels of antigen specific-IFN-γ (ng/ml) produced by maternal lymph node and spleen samples following stimulation for 4 days with NCA.



Levels of antigen specific-IFN-γ (ng/ml) produced by maternal lymph node and spleen samples following stimulation for 4 days with NCA.

Lymph node and spleen tissue samples were collected at post mortem examination and processed to determine levels of *Neospora*-specific proliferation. The processed cells were stimulated with NCA for 4 days (37^{o}C in a humidified 5% CO_2 atmosphere); ELISA were performed to determine the concentration of IFN-γ produced.

-▲- Group 1 -♦- Group 2 -■- Group 3 Error bars (S.E.)

7 Development of maternal and foetal immune responses in cattle following experimental challenge with *Neospora caninum* at day 210 of gestation

Paul M Bartley Frank Katzer, Mara S Rocchi, Stephen W Maley, Julio Benavides, Mintu Nath, Yvonne Pang, German Canton, Jackie Thomson, Francesca Chianini, and Elisabeth A Innes.

(2013)

Veterinary Research

7.1 Manuscripts main hypotheses

1 What is the clinical outcome of a subcutaneous (sc) challenge with *Neospora caninum* tachyzoites in pregnant cattle at late gestation?

2 Is there an increase in the expression of toll like receptor (TLR) 2 and TLR-9 in maternal and foetal lymphoid tissues following a challenge with *N. caninum* tachyzoites at late gestation?

3 What function do the maternal and foetal immune responses play in protection against a challenge with *N. caninum* at late gestation?

The results from this study demonstrate that though the clinical severity of an infection with *N. caninum* decreases as gestation progresses, the incidence of vertical transmission increases, with all of the challenged dams vertically transmitting the parasite to their foetuses. Though evidence of vertical transmission was seen in all challenged dams, there was no foetal mortality observed. During this study both dams and foetuses showed clear involvement of cell mediated, humoral and innate immune responses to *Neospora*.

This experiment showed just how rapidly vertical transmission can occur, even at day 14 post inoculation samples of foetal spleen showed evidence of significant antigen specific proliferation. This would suggest that the parasite must have crossed

the placenta within days of the initial challenge, to allow sufficient time for an antigen specific T-cell response to be produced.

As with much of the experimental work using large animals, this experiment would have benefited from larger group sizes, however due to the cost implications of buying and maintaining large animals for a prolonged period of time this was not possible. Meaning each challenge group had only 3 animals and the control data (3 animals) needed to be grouped. Though this study was the first to demonstrate a role of TLR-2 and TLR-9 in the immunity of cattle against *Neospora*, no function was ascribed to the TLR's. Further study is required to examine the cell types and activation pathways involved in the innate immunity during a *N. caninum* infection.

One disadvantage of a serial kill experiment is you only get to examine an animal at one time point, which only gives you a snapshot of the immune response. It would be interesting to sequentially examine the immune responses of the animals from immediately after inoculation until a defined endpoint (i.e. 56 Days pi), this could be done through PBMC or even through lymphatic cannulation. If the lymph node that drains the site of infection could be examined, it could yield important information on the initiation of the anti-*Neospora* immune response. This method could be used to examine differences the initiation and development of the immune responses at different stages of gestation, to try and improve our understanding of why at mid and late gestation the maternal immune response appears unable to limit the spread of the parasite and stop vertical transmission from occurring.

7.2 Author contributions

PMB, FK, MSR, SWM, JB, FC, JT and EAI were involved in all aspects of the experimental design and planning of the experiments. All experiments were approved by Moredun Research Institutes experimental ethics committee.

PMB, JBS and SWM inoculated all of the cattle, PMB and MSR collected routine blood samples for serological screening. PMB, FK, MSR, SWM, JB, FC, JT and EAI were all involved in the post mortem examinations of both dams and foetuses and for collecting all of the tissue samples used for immunological and histological analysis.

PMB maintained the Vero cells and *Neospora* tachyzoites in tissue culture and enumerated the cells and tachyzoites using a Neubauer haemocytometer and prepared all of the inocula (Vero cells and tachyzoites) used during the experiment. PMB and EAI performed lymphocyte stimulation assays on PBMC and lymph node and spleen samples from both dams and foetses, through the incorporation of ^{3}H thymidine and collected the cell stimulation assay supernatant samples. PMB analysed the supernatant samples from dams and foetuses by ELISA for the presence of the cytokines IL-4, IL-10, IL-12 and IFN-γ. PMB performed all RNA extractions, cDNA synthesis and SYBR green qPCR analysis for the presence of TLR-2, TLR-9 and GAPDH in both maternal and foetal spleen and lymph node samples. PMB collated all of the data and prepared all of the graphs and tables presented in the manuscript.

YP separated serum from clotted maternal and foetal blood samples from and performed all serological testing (IgG) using the commercially available IDEXX anti-*Neospora* IgG ELISA, while SWM, JB, GC and FC performed all histological and immunohistological analysis.

PMB, FK, MN and EAI drafted the original manuscript with contributions from all other authors

VETERINARY RESEARCH

RESEARCH Open Access

Development of maternal and foetal immune responses in cattle following experimental challenge with *Neospora caninum* at day 210 of gestation

Paul M Bartley[1*], Frank Katzer[1], Mara S Rocchi[1], Stephen W Maley[1], Julio Benavides[1,2], Mintu Nath[3], Yvonne Pang[1], Germán Cantón[1,4], Jackie Thomson[1], Francesca Chianini[1] and Elisabeth A Innes[1]

Abstract

This study examined the immunological responses of pregnant cattle and their foetuses following an experimental challenge with live *Neospora caninum* tachyzoites at day 210 of gestation. Animals were bled prior to and weekly throughout the experiment and sacrificed at 14, 28, 42 and 56 days post inoculation (dpi). At post mortem examination, samples of lymph nodes and spleen were collected from both dam and foetus for immunological analysis. Subcutaneous (sc) inoculation over the left prefemoral (LPF) lymph node of pregnant cattle at day 210 of gestation, led to the vertical transmission of parasites by 14 dpi, however no foetal deaths were observed in the infected animals. Foetuses from infected dams mounted *Neospora*-specific humoral and cell-mediated immune (CMI) responses by 14 dpi. These responses involved anti-*Neospora* IgG, antigen-specific lymphocyte proliferation, and the production of the cytokines IFN–γ, interleukin (IL)-4 and IL-10. There was also evidence of innate immunity during the response against *Neospora* from infected dams, with statistically significant ($p < 0.05$) increases in mean expression of toll like receptors (TLR)-2 on 56 dpi in maternal spleen, LPF, right prefemoral (RPF), left uterine (LUL) and right uterine (RUL) lymph nodes and TLR-9 in retropharyngeal (RLN), LPF and RPF lymph nodes from 28 dpi. Statistically significant ($p < 0.05$) increases in mean TLR-9 were detected in spleen samples from foetuses of infected dams, compared to the foetuses from control animals. Our results show that vertical transmission of the parasite occurred in all infected dams, with their foetuses showing effective *Neospora*-specific cell mediated, humoral and innate immune responses.

Introduction

Neospora caninum is recognised worldwide as a major cause of abortion and foetal death in farmed ruminants, mainly affecting cattle [1]; though sporadic cases have been reported leading to foetal deaths in sheep [2] and goats [3]. Infection with *Neospora* can occur either through vertical (transplacental) transmission of the parasite from dam to foetus [4], or through the ingestion of oocysts, shed by infected dogs [5] in contaminated feed, water and pasture (horizontal transmission). Current control strategies for bovine neosporosis mainly involve farm management and bio-security practices (reviewed by Dubey et al.) [1].

It has become clear from natural and experimental data that primary *Neospora* infections in cattle can lead to abortions and reproductive losses, with cattle infected with *Neospora* for the first time during pregnancy being 3–7 times more likely to abort than uninfected animals [6,7]. Animals can develop a level of natural immunity to the parasite, as cows previously naturally exposed to *Neospora* are less likely to abort following a secondary infection than pregnant animals with primary infections of the parasite [8]. In addition, multigravidae animals that have aborted due to neosporosis are less likely to abort due to neosporosis during subsequent pregnancies, compared to primigravidae cattle [9]. There is also

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experimental data [10] that demonstrates that exposure of cattle to *N. caninum* prior to pregnancy can protect against the vertical transmission of the parasite following an experimental challenge during mid gestation. However in naturally infected cows that were experimentally challenged on day 70 of gestation, the animals showed protection against foetopathy, but vertical transmission of the parasite still occurred [11]. These results suggest that a protective immune response may be induced that can protect against abortion, however, it may be more difficult to prevent endogenous vertical transmission of the parasite.

The gestational stage and immunological maturity of the foetus at time of infection with *N. caninum* are critical in determining the clinical outcome of infections in pregnant cattle [10,12,13]. Infection with *N. caninum* during early gestation leads to high levels of foetal mortality [14] and more severe pathology than infection of cattle with *N. caninum* at mid and late gestation [15]. While an experimental challenge with the parasite at mid gestation did not result in foetal mortality, it did however result in moderate pathology in the foetal central nervous system (CNS) and within the placenta [12]. The foetuses of cattle challenged at mid gestation were shown to be capable of mounting parasite-specific CMI and humoral responses [16]. Observed differences in the maternal immune response to *N. caninum* may have a profound effect on clinical outcome. A study by Bartley et al., [17], showed that in a group of pregnant cows given a similar challenge with *N. caninum*, some of the animals aborted their foetuses whereas other did not. These clinical differences could be related to the cell-mediated immune response of the animals to the parasite infection [17].

Neospora-specific cell mediated and innate immune responses are likely to be involved in protection against the parasite, in naturally [18] as well as experimentally infected animals [10,13,19,20]. Data from several studies has demonstrated that lymphocyte proliferation and interferon-γ (IFN-γ) responses are involved during an immune response to *N. caninum* [11,21-23], and that these responding immune cells tend to be $CD4^+$ T lymphocytes [20,24,25]. Less is known regarding the role of innate immune responses during bovine neosporosis. Work by Boysen et al. and Klevar et al. [26,27] reported that natural killer (NK) cells produced IFN-γ during early *Neospora* infection in calves. Work in mice infected with *N. caninum* has demonstrated that IFN-γ production is dependent on myeloid differentiation factor 88 signalling, in a mechanism triggered by interleukin-12 (IL-12) production in dendritic cells [28]. Increased toll like receptor (TLR)-2 expression leads to the maturation of antigen presenting cells such as macrophages and NK-cells and pro-inflammatory cytokine production [29]. These data show that the innate immune response may be important in the initiation of immune responses to *N. caninum*. Understanding of innate immunity to *N. caninum* will help to improve the design of effective vaccines, which rely on the induction of appropriate immune responses.

In this study the humoral, cell mediated and innate immune responses were examined in pregnant cattle and their foetuses experimentally challenged with live *N. caninum* (NC1 isolate) tachyzoites on day 210 of gestation. This is of particular interest as a primary exposure of cattle to *N. caninum* at late gestation can lead to high levels of transplacental transmission and the birth of persistently infected calves [30], which in turn are at risk of transmitting the parasite to their own offspring. This work will further the understanding of the role of both the maternal and foetal immune responses in cattle following an infection with *N. caninum* at late gestation and help to elucidate the mechanisms involved in disease pathogenesis and parasite transmission in bovine neosporosis.

Materials and methods

Animals, inoculum and experimental design

Fifteen pregnant cattle all being seronegative for *N. caninum*, *Toxoplasma gondii*, Bovine Viral Diarrhoea Virus (BVDV), infectious bovine rhinotracheitis and *Leptospira* spp., were divided into 2 groups (comprising of control cattle ($n = 4$) and animals inoculated with *N. caninum* ($n = 11$) with animals being sacrificed at 14, 28, 42 and 56 days post inoculation (dpi) (Table 1), a full description of the animals is given in Benavides et al. [30]. Parasites for the inoculum were prepared as previously described [31]. Briefly, *N. caninum* (NC1 isolate) tachyzoites were cultured within Vero cell monolayers. After 4 days the parasites were harvested by scraping the infected cell monolayers and releasing the tachyzoites into the supernatant. The parasites were harvested, counted and adjusted to the required concentration 2.5×10^8/mL in phosphate buffered saline (PBS). The 2 mL dose of parasites was inoculated sc into the animals over the left prefemoral lymph node (LPF) within 1 h of harvesting the tachyzoites from tissue culture. Uninfected Vero cells (5×10^7 per 2 mL dose in PBS) were used to inoculate each control animal over the LPF (Table 1). This dose of Vero cells was used, as it was the equivalent number of cells that was present in the parasite inocula.

Table 1 Experimental design

		Post mortem dpi/n =			
Group	Inoculum	14	28	42	56
Infected	5×10^8 NC1 Tachyzoites	3	3	3	2
Control	5×10^7 Vero cells	1	1	1	1

All animal procedures complied with the Animals (Scientific Procedures) Act 1986 and were approved by the Moredun Research Institute ethics committee.

Samples collected at post mortem

At post mortem examination, samples of LPF, right prefemoral lymph node (RPF), left uterine lymph node (LUL), right uterine lymph node (RUL), retropharyngeal lymph node (RLN) and spleen were collected from the dams. Samples collected from the foetuses were hepatic lymph node (HLN), mesenteric lymph node (MLN) and spleen. Tissue samples for immunological assays, cell proliferation and cytokine production were collected into sterile wash buffer comprising Hanks Buffered saline solution (HBSS) supplemented with 2% heat inactivated (ΔH) foetal bovine serum (FBS) (Labtech International, Ringmer, UK) and 100 IU/mL penicillin and 50 μg/mL streptomycin (Northumbria Biologicals, Cramlington, UK).

Blood was drawn from dams weekly and at post mortem by jugular venipuncture and from foetuses via the cordal vein, into non-heparinised vacutainer blood collection tubes, and allowed to clot; serum was then separated by centrifugation at $2000 \times g$ for 10 min and stored at −20 °C prior to enzyme linked immunosorbent assay (ELISA) analysis of anti-*Neospora* IgG.

Sub-samples of the maternal and foetal lymph nodes and spleen were collected for molecular analysis of innate immune responses. Samples were snap frozen on dry ice at post mortem examination, then stored at −80 °C prior to RNA extraction, cDNA synthesis and analysis by SYBR green quantitative polymerase chain reaction (qPCR).

Serology

Analysis of anti-*Neospora* IgG was performed using a commercially available ELISA kit (IDEXX, Chalfont St Peter, UK) following the manufacturer's instructions. Samples were considered positive with a sample/positive (S/P) value of ≥ 0.50. The S/P value was calculated using the optical density (OD) results and applying them to the formulae listed below:

$$\frac{\text{Sample OD Result} - \text{Negative Control OD Result}}{\text{Positive Control OD Result} - \text{Negative Control OD Result}}$$

Preparation of cells for immunological assays

Single cell suspensions of maternal and foetal lymph node and spleen samples were prepared using the method previously described by Bartley et al. [16]. Briefly; tissues were trimmed to remove excess fat and then chopped into small pieces. These pieces were resuspended in 10 mL of wash buffer and placed in a stomacher bag (Seward Medical, Northampton, UK) and homogenized for 10 s. The resultant cell suspension was decanted through a double thickness of sterile lens tissue into a sterile universal. The cells were washed twice by repeated centrifugation at $260 \times g$ before being resuspended at a final concentration of 2×10^6/mL in cell culture medium (CCM) (Iscoves modified Dulbeco's medium (IMDM) (Gibco, Paisley, UK) supplemented with 10% ΔH FBS and 100 IU/mL penicillin and 50 μg/mL streptomycin).

Cell proliferation assays

Single cell suspensions of both lymph node and spleen were treated as previously described by Bartley et al. [16]. In brief, equal volumes of cells (2×10^6/mL) and antigen were added in quadruplicate to 96-well round bottom plates (Nunc, Roskilde, Denmark). Water-soluble *N. caninum* tachyzoite antigen (NCA) [10] was used at a final protein concentration of 1 μg/mL, the T-cell mitogen concanavalin A (Con A) was used as a positive control at a final protein concentration of 5 μg/mL. Vero cell lysate antigen at 1 μg/mL final protein concentration was used as a control antigen and CCM alone was used as a negative control to determine the background level of cell proliferation. The cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere for 5 days. The cultures were pulsed with 18.5 kBq ^{3}H Thymidine/well (Amersham Biosciences, Little Chalfont, UK) for the final 18 h, before being harvested onto glass-fibre filters (Wallac, Turku, Finland); the cell associated radioactivity was determined using a microbeta Trilux liquid scintillation counter (Perkin Elmer, Wellesley, MA, USA).

Duplicate wells were prepared for each sample; after 4 days incubation at 37 °C in a humidified 5% CO_2 atmosphere; cell free supernatants were harvested and stored at −20 °C prior to analysis by ELISA to quantify cytokine production.

Cytokine responses

The concentrations of bovine cytokines; IFN-γ, IL-4, IL-10 and IL-12 present in the cell free supernatant were determined using commercially available cytokine capture ELISA; antibody pairs (Serotec, Oxford, UK) and serial dilutions of appropriate recombinant cytokines were used to create standard regression curves (Table 2).

The ELISA method used for all of the cytokines was based on that previously described by Kwong et al. [33]. In brief, 96-well ELISA plates (Greiner, Stonehouse, UK) were coated with a primary capture antibody (50 μL per well) (Table 2) and incubated at room temperature (RT) overnight. The plates were washed five times using PBS supplemented with 0.05% Tween 20 (PBS-T) between each step, with the exception of the final 3, 3', 5, 5'-tetramethylbenzidine (TMB) – sulphuric acid (H_2SO_4)

Table 2 Antibody pairs and standard ranges for bovine cytokines IFN-γ, IL-4, IL-10 and IL-12 ELISA

	Primary antibody (Working concentration (μg/mL))	Secondary antibody	Standard range	Reference
IFN-γ	CC330 (5 μg/mL)	CC302b (2 μg/mL)	5000 pg/mL –10 pg/mL	
IL-4	CC314 (5 μg/mL)	CC313b (2 μg/mL)	125.00 U/mL –0.488 U/mL	[32]
IL-10	CC318 (5 μg/mL)	CC320b (2 μg/mL)	11 U/mL –0.021 U/mL	[33]
IL-12	CC301 (1.25 μg/mL)	CC326b (1 μg/mL)	86.42 U/mL –0.013 U/mL	[34]

stage. The plates were blocked at room temperature for 1 h with PBS-T supplemented with 3 % bovine serum albumin (BSA). Samples and standards (50 μL each) were added and incubated at RT for 1 h. Plates were then coated with an appropriate secondary biotinylated antibody (Table 2) (diluted in PBS-T supplemented with 1% BSA) (50 μL per well) and incubated at RT for 1 h. Streptavidin- horseradish peroxidiase (HRP) (Dako Cytomation, Glostrup, Denmark) diluted 1:500 in PBS-T 1% BSA (50 μL/well) was added and incubated at RT for 45 min. Colour was developed by the addition of TMB substrate (Insight Biotech. Ltd., Wembley, UK) (100 μL/well) and incubated for 10–15 min in the dark. Reactions were stopped by adding 1 M H_2SO_4 (50 μL per well). The plates were read at 450/650 nm using a MRX II plate reader (Dynex, East Grinstead, UK). Doubling dilutions of known quantities of appropriate recombinant Ovine (rOv) or Bovine (rBo) cytokines (rOv IFN-γ, rBo IL-4, rBo IL-10 and rOv IL-12) (Moredun Research Institute, Edinburgh, UK) were used to generate a standard regression curve against which the test sample data was fitted. The rOv cytokines were used as standards for the ELISA as good cross reaction between rOv cytokines and bovine cells and antibodies has been previously demonstrated [34,35].

Extraction of RNA from maternal and foetal tissues

Samples collected and snap frozen on dry ice at post mortem were processed for RNA extraction as follows: approximately 1 g of frozen tissue was cut into small pieces and placed in a Precelys tissue homogeniser tube (Cepheid, Stretton Derbyshire, UK) containing 1.5 mL TRI reagent (Applied Biosystems, Carlsbad, CA, USA) and homogenised for 50 s at 6500 rpm using a Precelys 24 tissue homogeniser, (Cepheid, Stretton Derbyshire, UK). 700 μL of the resultant homogenate was split into each of two fresh microfuge tubes containing a further 300 μL TRI reagent. The samples were then processed to RNA as per manufacturer's instructions. The final RNA pellet was resuspended in 200 μL of RNase free water, the concentration of RNA was determined by spectrophotometry (Nanodrop ND1000); the samples were then stored at −80 °C prior to cDNA synthesis and qPCR.

cDNA synthesis from maternal and foetal RNA samples

Following the manufactures instructions, a commercially available high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA) was used to create 2 μg of cDNA per sample. Each reaction (20 μL) contained 2 μL 10× RT buffer, 0.8 μL dNTP (100 mM), 2 μL 10× random primers, 1 μL reverse transcriptase (50 U/μL), 1 μL RNase inhibitor, 3.2 μL DNase/RNase free water and 10 μL RNA (0.2 μg/μL); the reaction conditions for the cDNA synthesis reaction were 10 min at 25 °C, 120 min at 37 °C, 5 min at 80 °C then held at 4 °C. Following reverse transcription the cDNA was diluted to 400 μL in DNase / RNase free water and stored at 4 °C prior to qPCR analysis.

SYBR green qPCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), toll like receptor-2 (TLR) and TLR-9

To determine whether TLR-2 and TLR-9 expression is being up or down regulated during *N. caninum* infections we examined the expression of the TLRs in both maternal and foetal tissues at different stages following challenge, using the SYBR green qPCR primers and basic protocol described by Menzies and Ingham [36]. All samples were analysed in triplicate, each reaction (20 μL) consisted of 10 μL (2× Fast SYBR green master mix) (Applied Biosystems, Carlsbad, CA, USA), 1 μL each forward and reverse primer (10 pmol), 4 μL DNase/RNase free water and 4 μL cDNA. Analysis was performed under standard conditions including a dissociation step (ABI prism 7000 using sequence detection software (SDS) (v1.2.3) (Applied Biosystems, Carlsbad, CA, USA). The levels of TLR expression were normalised against a reference gene GAPDH, which has been previously shown to be constitutively expressed in bovine tissues [36].

Statistical analysis

To take into account the increased variability at higher mean values, the maternal and foetal proliferation (cpm) data and cytokine assay data (IFN-γ) were transformed by logarithmic transformation (base 10) prior to the analysis. The TLR-2 and TLR-9 data were adjusted against GAPDH. Separate linear models were fitted to the maternal and foetal proliferative responses, cytokine responses and TLR expression. Due to lack of replication of the control group at each time point, data on the control group were pooled across all time points to estimate the variability. The models included treatment group (comprising five levels: infected group at each of four

time points and the control group) as an explanatory variable. The model assumes normal distribution for the errors on the analysis scale and this was checked using appropriate plots. The overall statistical significance of the treatment group was evaluated using the *F*-statistic. If the *F*-statistic was statistically significant ($p \leq 0.05$), two-sided p-values for multiple comparisons between the means of the infected group at each time point and the pooled mean of the control group were obtained; these p-values were then adjusted using a False Discovery Rate (FDR) approach [37] to take into account the problems arising from multiple comparisons. The adjusted *p*-value, denoted in this paper as p_f, summarises the strength of evidence for there being a genuine difference in a way analogous to a standard *p*-value but allowing multiple testing across different times. Difference in the median maternal serology IgG values between the infected and control group across each time point was assessed using a two sample non-parametric Mann–Whitney test. To take into account multiple comparisons at all time points, the FDR approach as discussed above was used to adjust the p-values. In general, the statistical significance is considered at 5% level ($p_f < 0.05$), though for some instances where $p < 0.05$ but not p_f, both p and p_f values are provided. All statistical analyses were carried out using the R software version 2.15.2 (R Development Core Team, 2012).

Results

Proliferative responses from lymph nodes and spleen

Maternal

Infected dams showed increased mean levels of antigen-specific proliferation (counts per minute (cpm) on $\log_{10}$ scale) (Additional file 1) from most tissues examined, compared to the control animals at all the time points, though for most samples no statistically significant differences were seen. On 14 dpi statistically significantly ($p = 0.034$) increased mean levels of antigen-specific proliferative responses were observed in the spleen samples from infected animals, compared to control animals. At the same time point, significantly lower ($p = 0.023$) mean levels of proliferation were observed in the RPF of infected animals (mean, 2.56; 95% CI, 1.73 to 3.40) compared to control animals (mean, 3.99; 95% CI, 3.16 to 4.82). However, after allowing for multiple testing, the adjusted p-values for these mean comparisons were not statistically significant for either spleen ($p_f = 0.137$) or RPF ($p_f = 0.09$).

Foetal

The cell proliferation responses from the foetuses are illustrated in Figure 1. Mean levels of antigen-specific proliferation (cpm on $\log_{10}$ scale) in the spleen were statistically significantly higher in the infected foetuses on 14 dpi ($p_f = 0.032$), 28 dpi ($p_f < 0.001$), 42 dpi ($p_f < 0.001$) and on 56 dpi ($p_f < 0.001$) compared to control foetuses. Significantly increased mean levels of proliferation were observed in HLN samples from infected foetuses on 42 dpi ($p_f = 0.038$) and 56 dpi ($p_f = 0.038$) compared to the control foetuses. There was no evidence of a difference in the mean levels of antigen-specific proliferation in foetal MLN tissue from either group (Figure 1A-D).

Cytokine responses of lymph node and spleen

Maternal

IFN-γ

Increased mean levels of *Neospora*-specific IFN-γ (ng/mL on $\log_{10}$ scale) (Additional file 2) were observed in most maternal lymph nodes and spleen samples from infected dams compared to control animals at all the time points. Statistically significantly higher mean levels of IFN-γ were observed on 14 dpi ($p_f = 0.021$) in the spleen from infected animals compared to the control animals, while on 56 dpi, mean levels of antigen-specific IFN-γ in spleen samples from infected animals were lower than those seen in the control animals with marginal statistical significance ($p_f = 0.060$).

IL-4

Mean levels of antigen-specific IL-4 were higher in all maternal samples from infected animals compared to the control animals, at all of the time points tested (Additional file 3). Statistically significantly increased mean levels of antigen-specific IL-4 were demonstrated on 14 dpi in the spleen ($p = 0.028$) and LPF ($p = 0.012$) of the infected animals compared to the control animals However, when allowing for multiple testing, the mean differences between treatment groups were not statistically significant for either spleen or LPF ($p = 0.130$, $p = 0.121$ respectively).

IL-10

Demonstrable antigen-specific IL-10 was only found in the spleen sample from the control animal on 28 dpi, all other lymph node and spleen samples from the control animals were below the detection threshold of the ELISA. In the infected animals, on 14 dpi demonstrable levels of antigen-specific IL-10 were seen in spleen samples from 2 of 3 animals. On 28 dpi, IL-10 was produced in 3 of 3 samples of spleen and LUL. On 42 dpi (2/3) spleen, (1/3) RUL and RLN samples were shown to be producing IL-10. On 56 dpi, IL-10 was only demonstrable in the LUL from 1 of 2 of the infected animals.

IL-12

All the maternal lymph node and spleen samples tested from the control animals were below the detection

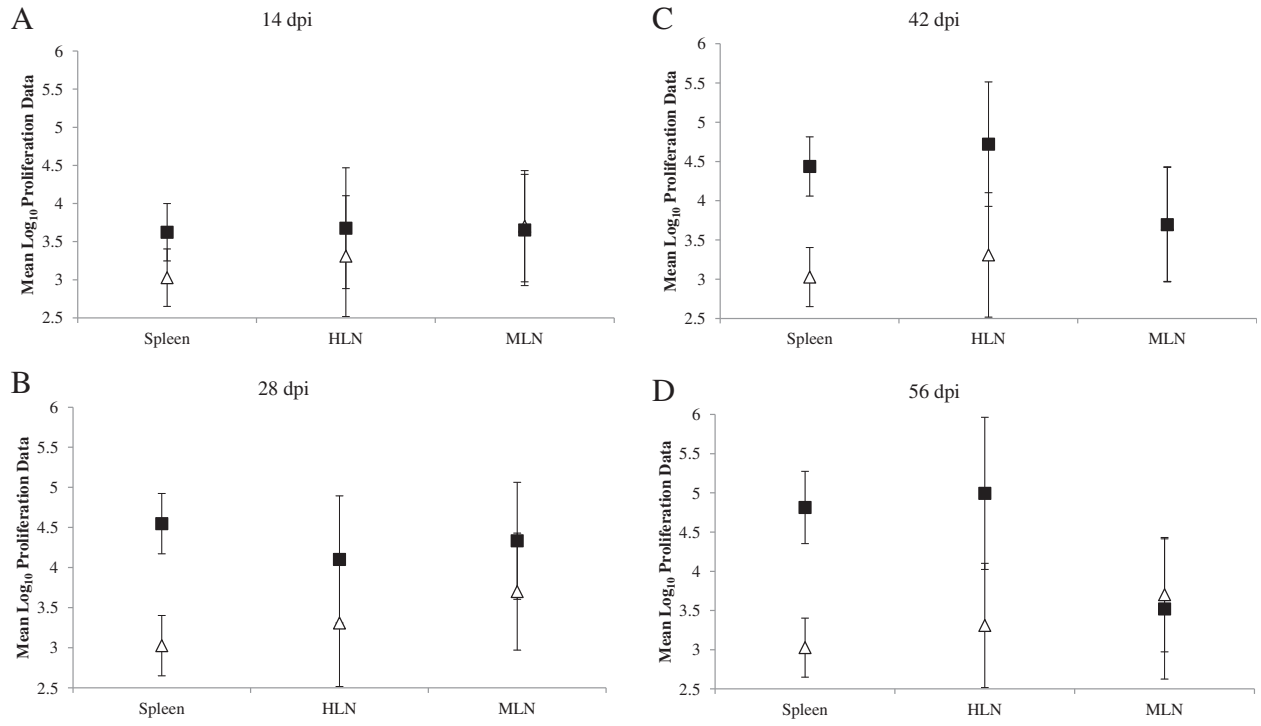

Figure 1 Foetal lymph node and spleen proliferation responses following stimulation with NCA for 5 days. Samples of foetal hepatic lymph node (HLN), mesenteric LN (MLN) and and spleen were collected at post mortem examination. Samples were stimulated with NCA for 5 days (37° C in a humidified 5% CO_2 atmosphere), with 18.5 kBq ^{3}H Thymidine / well being added for the final 18 h, before being harvested onto glass-fibre filters. The data were log-transformed (base 10) before analysis by a linear model. The predicted mean responses (counts per minute (cpm) on $\log_{10}$ scale) (Infected -■-, Control -Δ-) and 95% confidence intervals (error bars) of foetal spleen and lymph node on **(A)** 14 dpi, **(B)** 28 dpi, **(C)** 42 dpi and **(D)** 56 dpi are presented).

threshold of the ELISA for antigen-specific IL-12. Demonstrable levels of IL-12 were seen in maternal spleen samples from infected animals on 14 and 28 dpi only.

Foetal

IFN-γ

The results from the IFN-γ ELISA are illustrated in Figure 2A-D. Spleen samples from the foetuses in infected dams showed increased mean levels of antigen-specific IFN-γ production (ng/mL on $\log_{10}$ scale) compared to the foetuses from control animals on 14, 28, 42 and 56 dpi. These mean differences in IFN-γ levels of the spleen between control and infected foetuses were statistically significant at all time points ($p_f = 0.018$, $p_f = 0.028$, $p_f = 0.028$ and $p_f = 0.018$ for 14, 28, 42 and 56 dpi respectively) (Figure 2A-D). Samples of HLN and MLN from infected foetuses were generally demonstrated increased mean levels of antigen-specific IFN-γ production compared to the foetuses from the control animals at all time points, though these differences were not statistically significant (Figure 2A-D).

IL-4

The infected foetuses showed an increased mean level of production of antigen-specific IL-4 in the spleen compared to the control foetuses on 28, 42 and 56 dpi ($p_f = 0.012$, $p_f = 0.052$ and $p_f = 0.012$ respectively) (Additional file 4). On 14 dpi the mean MLN response from the infected foetuses was significantly higher ($p = 0.024$) compared to the control foetuses. However, this mean difference was not statistically significant once allowance was made for multiple testing ($pf = 0.114$). No statistically significant differences were observed between the control and infected foetuses for mean levels of antigen-specific IL-4 produced by foetal HLN samples.

IL-10

All the samples tested for the control foetuses were below the detection threshold of the ELISA. However, demonstrable antigen-specific IL-10 was seen in spleen samples from the infected foetuses at all time points, on 14 dpi one of three foetuses produced IL-10 and on 28 dpi two of three foetuses produced IL-10. On 42 dpi, all infected foetal spleen samples (3 / 3) produced demonstrable antigen-specific IL-10. On 56 dpi, spleen and HLN samples from both infected foetuses produced demonstrable levels of antigen-specific IL-10. The mean levels of IL-10 produced by the foetal samples were comparable with maternal samples (data not shown).

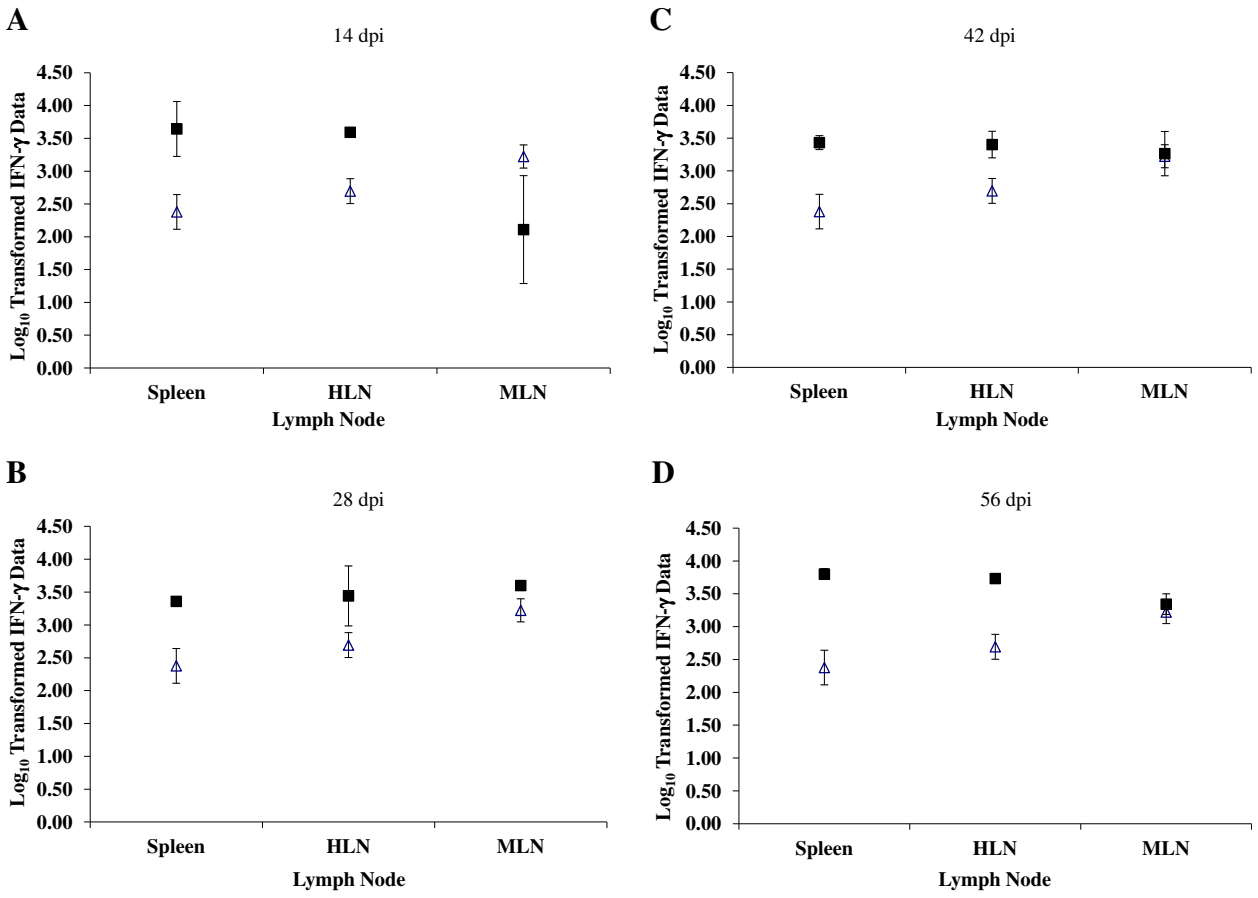

Figure 2 Foetal lymph node and spleen IFN-γ responses following stimulation with NCA for 4 day. Foetal hepatic lymph node (HLN), mesenteric LN (MLN) and spleen samples were collected at post mortem examination. Following stimulation with NCA for 4 days (37° C in a humidified 5% CO_2 atmosphere) cell free supernatants were harvested, ELISA were performed to determine the concentration of IFN-γ (ng/mL) produced. The data were log-transformed (base 10) before analysis by a linear model. The predicted mean responses (ng/mL on log_{10} scale) (Infected -■-, Control -Δ-) and 95% confidence intervals (error bars) of foetal spleen and lymph node on **(A)** 14 dpi, **(B)** 28 dpi, **(C)** 42 dpi, **(D)** 56 dpi.

IL-12

All the samples tested for control foetuses were below the ELISA detection threshold IL-12 at all time points tested. Spleen samples from infected foetuses (1/3) produced demonstrable levels of IL-12 on 14 dpi. On 28 and 42 dpi, the levels of antigen-specific IL-12 were below detectable levels. However, on 56 dpi, IL-12 was demonstrated from the spleen samples of both infected foetuses. The mean levels of IL-12 produced by the foetal samples were comparable with maternal samples (data not shown).

Expression of TLR-2 and TLR-9 in lymph node and spleen

Maternal

TLR-2

Levels of TLR-2 expression were examined at all time points in both control and infected animals. The infected dams demonstrated increased mean levels of TLR-2 expression (pg) in all tissues compared to the control dams at almost all time points. On 56 dpi, the infected dams demonstrated statistically significantly increased mean levels of TLR-2 expression in the LPF and RPF compared to the control dams ($p_f = 0.004$ and $p_f < 0.001$ respectively) (Figure 3C), while the spleen, LUL and RUL samples from the infected animals were all shown to be producing statistically significantly higher mean levels of TLR-2 ($p = 0.035$, $p = 0.024$ and $p = 0.018$ respectively) than the control animals (Figure 3A-C).

TLR-9

Levels of TLR-9 expression were examined at all time points in both control and infected animals. Infected animals demonstrated increased mean levels of TLR-9 expression (pg) from day 28 onwards in all tissues tested compared to control animals. On 28 dpi statistically significant mean differences were seen between control and

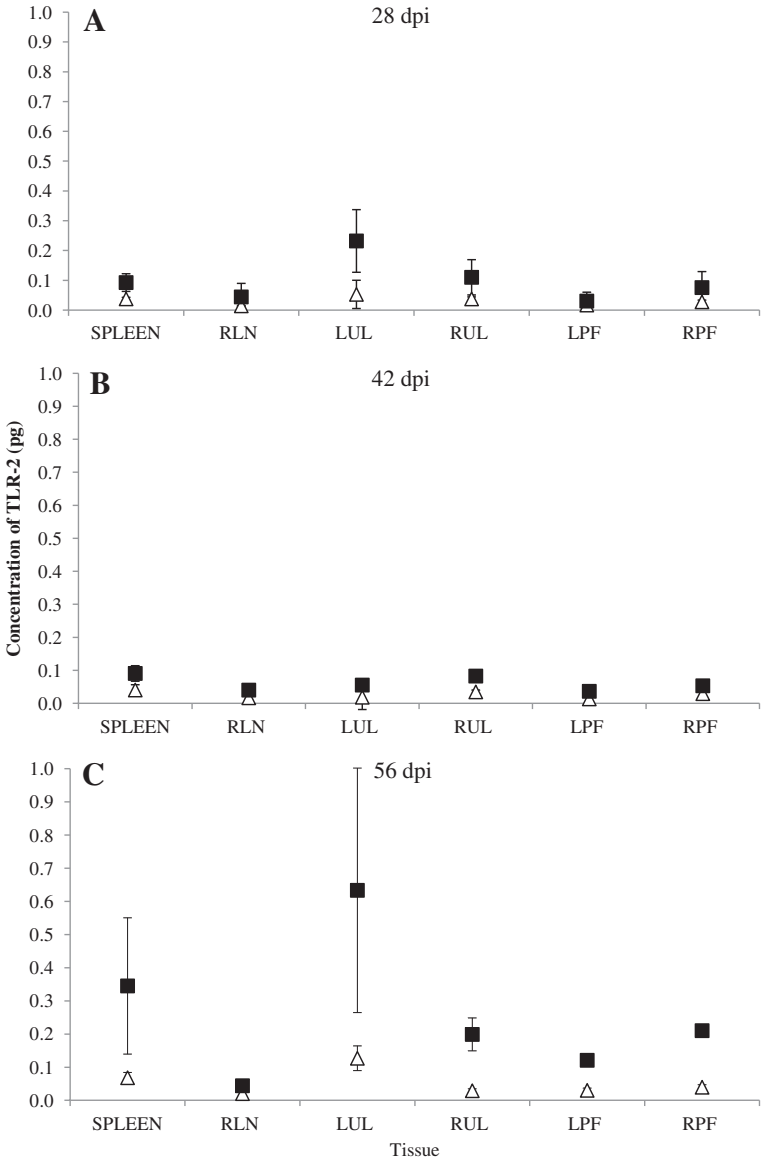

Figure 3 Maternal lymph node and spleen TLR-2 expression. Samples of maternal left prefemoral lymph node (LPF), right prefemoral lymph node (RPF), left uterine lymph node (LUL), right uterine lymph node (RUL), retropharyngeal lymph node (RLN) and spleen were collected at post mortem examination and snap frozen on dry ice. RNA was extracted and used to synthesise cDNA. Levels of expression of TLR-2 were examined with data being normalised against GAPDH expression(pg). The predicted mean responses (pg) (Infected -■-, Control -Δ-) and 95% confidence intervals (error bars) of maternal spleen and lymph node on **(A)** 28 dpi, **(B)** 42 dpi, **(C)** 56 dpi.

infected animals, in RLN ($p = 0.016$), RPF ($p = 0.042$) and LPF ($p = 0.051$) and in spleen ($p = 0.034$) on 42 dpi. While on 56 dpi, LPF ($p_f = 0.027$) and RPF ($p_f = 0.028$) from infected animals demonstrated statistically significantly higher mean levels of expression of TLR-9 compared with the control animals (Figure 4A-C).

Foetal

TLR-2

The foetuses from infected animals generally demonstrated increased mean levels of TLR-2 expression (pg) (Additional file 5) in samples of spleen, HLN and MLN compared to the control foetuses. However, these mean differences were not statistically significant. The mean levels of TLR-2 expression seen in the foetal samples were comparable to those observed in the adult samples.

TLR-9

When the mean levels of expression of TLR-9 (pg) were examined, statistically significantly increased expression was only observed in the spleen ($p = 0.025$) of the

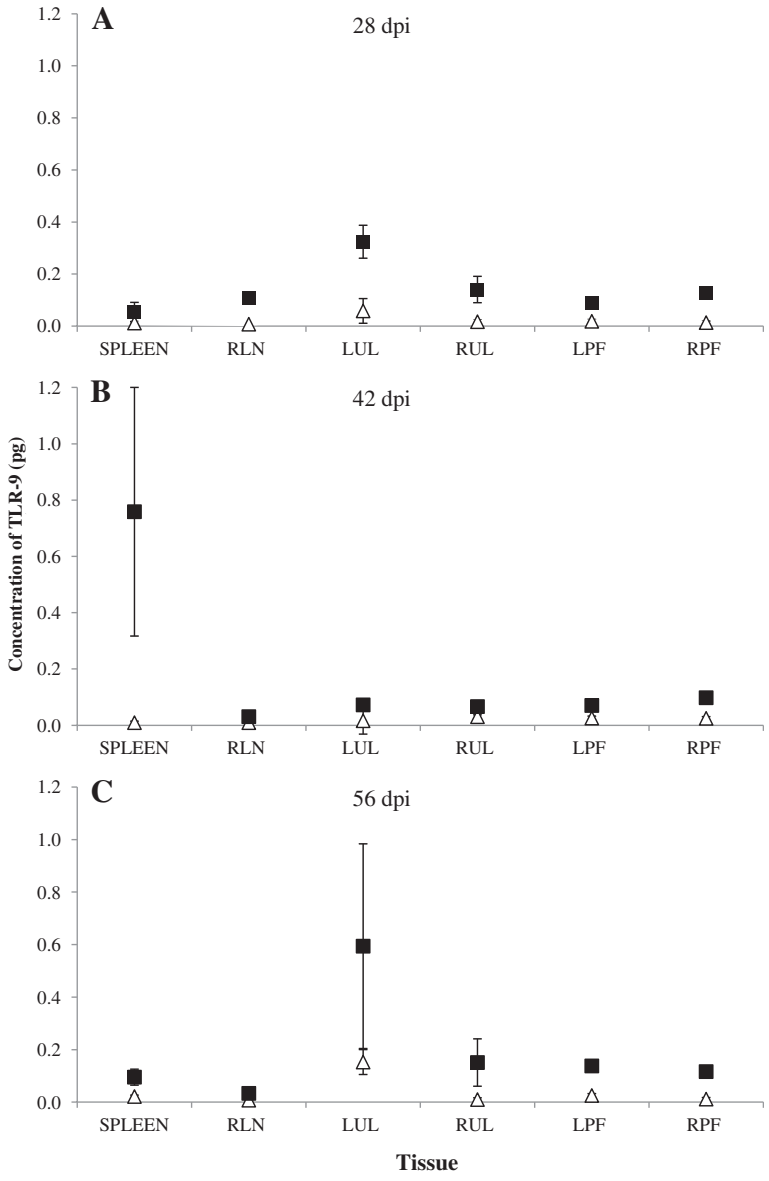

Figure 4 Maternal lymph node and spleen TLR-9 expression. Samples of maternal left prefemoral lymph node (LPF), right prefemoral lymph node (RPF), left uterine lymph node (LUL), right uterine lymph node (RUL), retropharyngeal lymph node (RLN) and spleen were collected at post mortem examination and snap frozen on dry ice. RNA was extracted and used to synthesise cDNA. Levels of expression of TLR-9 were examined with data being normalised against GAPDH expression (pg). The predicted mean responses (pg) (Infected -■-, Control -Δ-) and 95% confidence intervals (error bars) of maternal spleen and lymph node on **(A)** 28 dpi, **(B)** 42 dpi, **(C)** 56 dpi.

infected foetuses on 42 dpi compared to the control foetuses. However, the mean difference was not statistically significant when adjusted for multiple testing (p_f = 0.101). Generally, the mean expression of TLR-9 of infected foetuses was higher compared to control animals in the spleen, HLN and MLN tissues at all time points. The mean expression of TLR-9 was consistently higher for all samples on 14 dpi, with the spleen demonstrating the highest levels of expression from any of the samples tested (data not shown).

Serology

Maternal

Following inoculation with *N. caninum* tachyzoites all infected dams seroconverted between 7 and 14 dpi (Figure 5). The mean levels of IgG peaked on 21 dpi, the levels of anti-*Neospora* IgG remained elevated throughout the rest of the experimental period. The mean levels of anti-*Neospora* IgG in all control animals remained below the 0.50 cut off throughout. The infected animals demonstrated statistically significantly higher median levels of

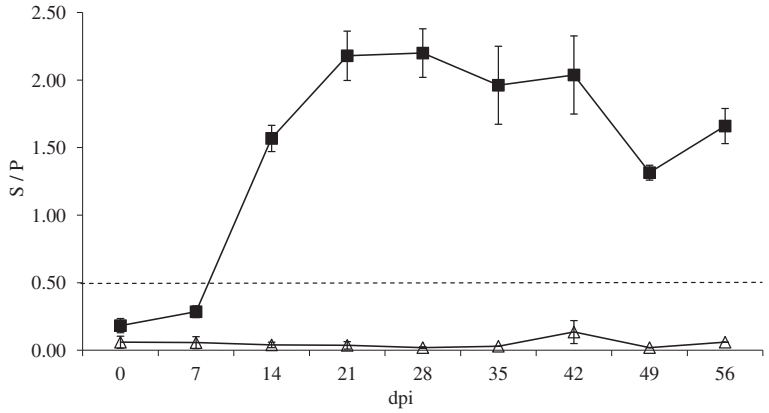

Figure 5 Maternal serology (IgG ELISA) results. Blood was drawn from the dams by jugular venipuncture into non-heparinised vacutainer blood collection tubes and allowed to clot. Serum was separated by centrifugation at 2000 × *g* for 10 min and stored at −20 °C prior to analysis of anti-*Neospora* IgG using a commercially available ELISA kit (IDEXX). Samples were considered positive with a sample / positive (S / P) value of ≥ 0.50. The S / P value was calculated using the optical density (OD) results. The estimates of means (Infected -■-, Control -Δ-) and standard of means (error bars) are presented.

anti-*Neospora* IgG on 7, 14, 21 and 28 dpi; ($p_f = 0.054$, $p_f = 0.032$, $p_f = 0.032$ and $p_f = 0.032$ respectively) than the control animals. The median levels of anti-*Neospora* IgG expression in the infected animals remained elevated above the control animals for the remainder of the experiment, though these were not found to be statistically significant.

Foetal

No anti-*Neospora* IgG was demonstrable in the samples collected from infected foetuses on 14 and 28 dpi. However from 42 dpi onwards all foetuses from infected dams tested positive for anti-*Neospora* IgG (Figure 6). The levels of anti-*Neospora* IgG produced by the infected foetuses were comparable to those seen in the adult infected animals. None of the samples tested from control foetuses were considered positive for anti-*Neospora* IgG as they remained below the 0.50 cut off throughout. No statistical comparisons were made due to the properties of the data.

Samples collected from control animal

Following post mortem examination, samples from the control animal sacrificed on 14 dpi were shown to be positive by *N. caninum* specific ITS1 PCR. Further examination of these samples using primers designed against regions of micro satellite DNA demonstrated

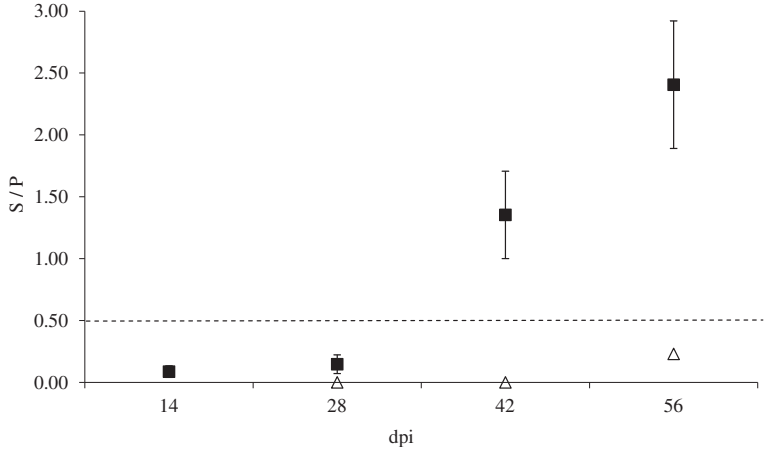

Figure 6 Foetal serology (IgG ELISA results). Blood was drawn from the foetuses at the post mortem examination into non-heparinised vacutainer blood collection tubes and allowed to clot. Serum was separated by centrifugation at 2000 × *g* for 10 min and stored at −20 °C prior to analysis of anti-*Neospora* IgG using a commercially available ELISA kit (IDEXX). Samples were considered positive with a sample / positive (S/P) value of ≥ 0.50. The S/P value was calculated using the optical density (OD) results. The estimates of means (Infected -■-, Control -Δ-) and standard of means (error bars) are presented.

that the animal was infected prior to the experiment and the infection was due to a strain of *N. caninum* distinct to NC1. As a result of these findings this animal and its foetus were removed from all analysis. Full details of the animal are given in Benavides et al. [30]. This animal was serologically negative for anti-*Neospora* IgG (using IDEXX ELISA) and had no demonstrable antigen specific responses in the cells from the spleen or any of the lymph nodes tested. All the samples tested from the foetus derived from this dam were also immunologically and PCR negative.

Discussion

At late gestation (day 210), the foetuses are capable of mounting *Neospora* specific cell mediated and humoral immune responses, involving lymphocyte proliferation, antigen-specific IFN-γ, IL-4 and IL-10 production as well as anti-*Neospora* IgG antibodies. This study has also examined the innate immune response during an experimental infection of cattle, showing significantly increased levels of expression of TLR-2 and TLR-9 in a number of maternal and foetal lymphoid tissues including the spleen, uterine and pre-femoral lymph nodes during the course of the infection.

Maternal CMI responses

Following challenge there were significant increases in cellular proliferation as well as IFN-γ and IL-4 production on 14 dpi in the spleen and peripheral lymph nodes of infected dams and their foetuses, The presence of a CMI response by 14 dpi correlates well with the observation of a circulating parasitaemia observed in all infected dams between 8 – 14 dpi [30], thus providing a source of antigen for immunological priming to take place. The presence of parasites and parasite antigens is likely to lead to the rapid initiation of an immune response. Our findings agree with work by Rosbottom et al. [38] where pregnant cattle experimentally challenged with *N. caninum* on day 210 of gestation demonstrated increased numbers of CD4+ T-cells and increased expression of IFN-γ and IL-4 mRNA in PBMC, 1–2 weeks following challenge.

Toll like receptors

Our study shows that TLR-2 and TLR-9 are up regulated in the spleen and lymph nodes of infected animals and their foetuses during *N. caninum* infection. Significant increases in the levels of TLR-2 were not seen in maternal tissues until 56 dpi and TLR-9 on 28 dpi. This is later than we would have expected, as much of the current data regarding TLR function shows they are involved in the initiation and activation of immune responses. Work by Werling et al. [39] in cattle has shown strong TLR-2 signalling to be associated with monocytes and other antigen presenting cells (APC), while TLR-9 signalling was associated with bovine dendritic cells (DC) and B-cells. Similar finding were observed in mice by where TLR-2 was associated with APC maturation and pro-inflammatory cytokine production [28,29]. Cantón et al. [40] observed large numbers of phagocytic cells (in particular macrophages) in resolving lesions in the placenta of infected animals from this experiment from 42 dpi onwards, While on 42 dpi Benavides et al. [30] described lesions in foetal lung and liver characterised by the infiltration of macrophages, lymphocytes and plasma cells. The presence of these cells in the circulatory system may account for the increased levels of TLR-2 expression seen in the spleens of the infected compared to control animals. The increase in TLR-9 expression may be a consequence of increased B-cell activity following the seroconversion of the infected animals and the production of anti-*Neospora* IgG.

Humoral immune responses

Infected dams and their foetuses developed strong anti-*N. caninum* humoral responses following experimental challenge with the parasite, which continues to suggest a role for antibodies in a protective immune response [41]. Antibody responses were seen in the infected dams from 7 dpi onwards, this coincided with high levels of circulating parasites, however in the foetuses anti-*Neospora* antibodies were not observed until 42 dpi. The delay in the generation of a foetal humoral response could be a consequence of the low numbers of parasites crossing the placenta and actively invading the foetuses, until 28 dpi all foetal tissues were PCR negative [30] Though antibodies against *N. caninum* have not been shown to have a definitive role in protection; it is widely believed that they are involved in extracellular tachyzoite neutralisation. Work by Eperon et al. [42] demonstrated increased susceptibility to infection with *N. caninum* of B-cell deficient C57BL/6 μMT mice. While work on the closely related parasite *Toxoplasma* has demonstrated roles for antibodies in parasite killing by large granular lymphocytes [43] and in opsonisation and intracellular killing of parasites by mononuclear phagocytes [44].

Foetal immune response

Due to the nature of the ruminant cotyledonary placentae (syndesmochorial placentation), any immune responses detected in the foetus are likely to be induced by an active infection in utero [45]. Under normal circumstances a cotyledonary placentae does not allow the transfer of maternal immune factors including antibodies and cytokines. During our study foetal cell mediated immune responses were detected at 14 dpi, which suggests that even during the early stages of the infection

the parasites crossing the placenta are being dealt with effectively by the foetal immune response, before parasite induced pathology can occur The mild placental pathology being observed, may also be due to there being fewer parasites multiplying in the foetus, therefore there are fewer parasites reinvading the placenta. A reason for this may be that when the parasites reach the placenta they initiate a local maternal immune response. During our experiment increased levels of proliferation and IFN-γ production were seen in the uterine lymph nodes of infected animals compared to the controls at 14 dpi. This may in the first instance limit parasite establishment and multiplication reducing the severity of necrosis and pathology in the tissues around the placenta [45], allowing for more efficient parasite clearing. Coupled with the developing foetal immunity could potentially lead to the scarcity of lesions containing parasite antigen found in the CNS of the infected foetuses. A previous experimental study in cattle [46] showed comparable results to this current study; where cattle infected with *N. caninum* (NC Liverpool) at late gestation showed no foetal mortality, though mild placental pathology was observed and parasite DNA was only found sporadically in foetal brain, lung and skeletal muscle.

There is still limited information regarding the development of foetal immune responses during bovine *Neospora* infections. Following infection at early gestation (Day 70 of gestation) [17] showed that no *Neospora*-specific CMI responses were generated, though the foetuses were capable of lymphocyte proliferation and IFN-γ, IL-4, IL-10 and IL-12 production following mitogenic stimulation with Con A from day 84 of gestation onwards, however by mid gestation the foetal immune system is more capable of mounting antigen specific humoral and cell-mediated immune responses [45]. Experimental infections in cattle with *Neospora* at mid gestation (Day 140 of gestation) have shown significant increases in IFN-γ, IL-10 and tumour necrosis factor (TNF) expression [19] and *Neospora*-specific CMI and humoral responses [16] in infected foetuses. While, Andrianarivo et al. [21] showed *Neospora*-specific immune responses in foetal PBMC at around day 220 of gestation.

During our experiment antigen-specific immune responses were present in the foetuses on 14 dpi demonstrating that parasites crossed the placenta, infected the foetuses and induced an immune response. The numbers of parasites in foetal tissues is likely to be very low, as no positive PCR results were seen at 14 dpi [30]. The foetal spleen processes large volumes of blood, this may result in it potentially trapping parasites as well as circulating leukocytes, allowing more rapid presentation of parasite antigens leading to proliferation and cytokine production before the HLN and MLN have started to respond. The presence of parasite DNA and strong cell mediated immune responses being found in all foetuses would suggest that though the parasite crossed the placenta, the foetal immune response was sufficiently robust to control the parasite resulting in the survival of all infected foetuses to the end of the experimental period.

Conclusions

The results from this study have demonstrated that following an experimental sc challenge of pregnant cattle with live *N. caninum* tachyzoites on day 210 of gestation; both dams and foetuses mounting *Neospora*-specific cell mediated, humoral and innate responses. These results show that the stage of gestation is important to disease outcome, with the increasing immunological maturity of the foetus limiting the clinical severity of the infection compared to *Neospora* infections occurring earlier in gestation. The results show that infections with *Neospora* at late gestation may lead to congenitally infected but otherwise clinically normal calves [30]. Experimental challenges of pregnant cattle with *N. caninum* tachyzoites allows the detailed study of the host – parasite relationship in bovine neosporosis in a controlled environment, thus improving our understanding of the pathogenesis of the disease.

Additional files

Additional file 1: Mean Log_{10} proliferation data from maternal lymph node and spleen samples following stimulation with NCA for 5 days. Mean Log_{10} proliferation data from maternal lymph node and spleen samples following stimulation with NCA for 5 days. Samples of maternal lymph node and spleen were collected at post mortem examination. Samples were stimulated with NCA for 5 days (37 °C in a humidified 5% CO_2 atmosphere), with 18.5 kBq ^{3}H Thymidine / well being added for the final 18 h, before being harvested onto glass-fibre filters. The data was Log_{10} transformed before analysis by a linear model. (A) 14 dpi, (B) 28 dpi, (C) 42 dpi, (D) 56 dpi. Infected ■, Control Δ (Error Bars = upper (U) & lower (L) 95% confidence intervals (CI)).

Additional file 2: Mean Log_{10} transformed IFN-γ data from maternal lymph node and spleen samples following stimulation with NCA for 4 days. Mean Log_{10} transformed IFN-γ data from maternal lymph node and spleen samples following stimulation with NCA for 4 days. Maternal lymph node and spleen samples were collected at post mortem examination. Following stimulation with NCA for 4 days (37 °C in a humidified 5% CO_2 atmosphere) cell free supernatants were harvested, ELISA were performed to determine the concentration of IFN-γ produced. The data was Log_{10} transformed before analysis by a linear model. (A) 14 dpi, (B) 28 dpi, (C) 42 dpi, (D) 56 dpi. Infected ■, Control Δ (Error Bars = U & L 95% CI).

Additional file 3: Concentration of IL-4 in maternal lymph node and spleen samples following stimulation with NCA for 4 days. Concentration of IL-4 in maternal lymph node and spleen samples following stimulation with NCA for 4 days. Maternal lymph node and spleen samples were collected at post mortem examination. Following stimulation with NCA for 4 days (37 °C in a humidified 5% CO_2 atmosphere) cell free supernatants were harvested, ELISA were performed to determine the concentration of IL-4 produced. (A) 14 dpi, (B) 28 dpi, (C) 42 dpi, (D) 56 dpi. Infected ■, Control Δ (Error Bars = U & L 95% CI).

Additional file 4: Concentration of IL-4 in foetal lymph node and spleen samples following stimulation with NCA for 4 days. Concentration of IL-4 in foetal lymph node and spleen samples following stimulation with NCA for 4 days. Foetal lymph node and spleen samples were collected at post mortem examination. Following stimulation with NCA for 4 days (37 °C in a humidified 5% CO_2 atmosphere) cell free supernatants were harvested, ELISA were performed to determine the concentration of IL-4 produced. (A) 14 dpi, (B) 28 dpi, (C) 42 dpi, (D) 56 dpi. Infected ■, Control Δ (Error Bars = U & L 95% CI).

Additional file 5: Levels of expression of TLR-2 in foetal spleen, HLN and MLN samples. Levels of expression of TLR-2 in foetal spleen, HLN and MLN samples. Samples of foetal lymph node and spleen were collected at post mortem examination and snap frozen on dry ice. RNA was extracted and used to synthesise cDNA. Levels of expression of TLR-2 were examined with data being normalised against GAPDH expression, results are expressed in pg. **(A)** 28 dpi, **(B)** 42 dpi, **(C)** 56 dpi. Infected ■, Control Δ (Error Bars = U & L 95% CI).

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
PMB, FK, JB and EAI made substantial contributions to the conception and design. PMB, MSR, JT, YP, GC, SWM, FC and EAI were involved in the acquisition of data. PMB and MN were involved in the analysis of the data. PMB, FK, MN and EAI have been involved in the drafting and critical review of the manuscript. All authors read and approved the final manuscript.

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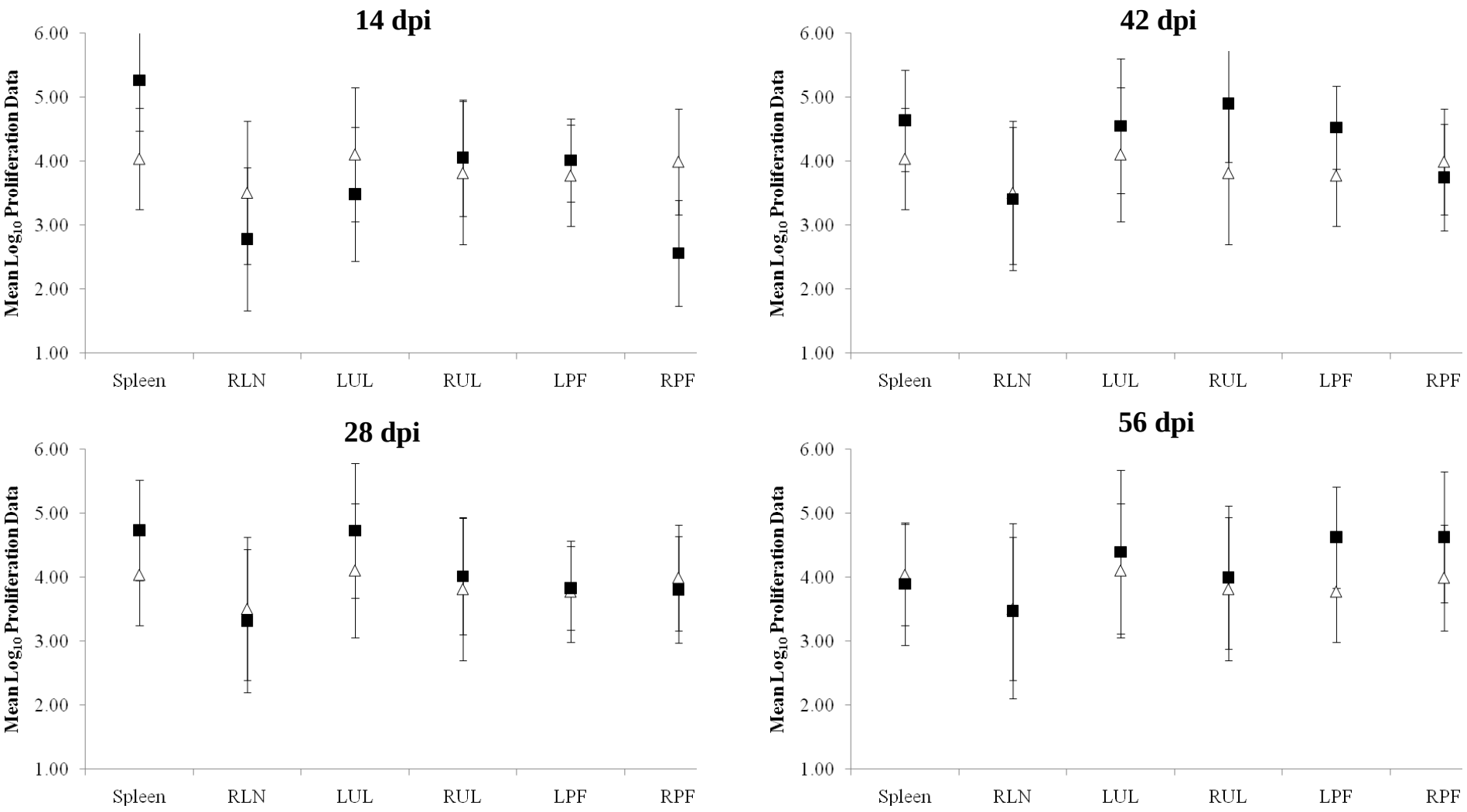
Additional File 1. Mean Log_{10} proliferation data from maternal lymph node and spleen samples following stimulation with NCA for 5 days

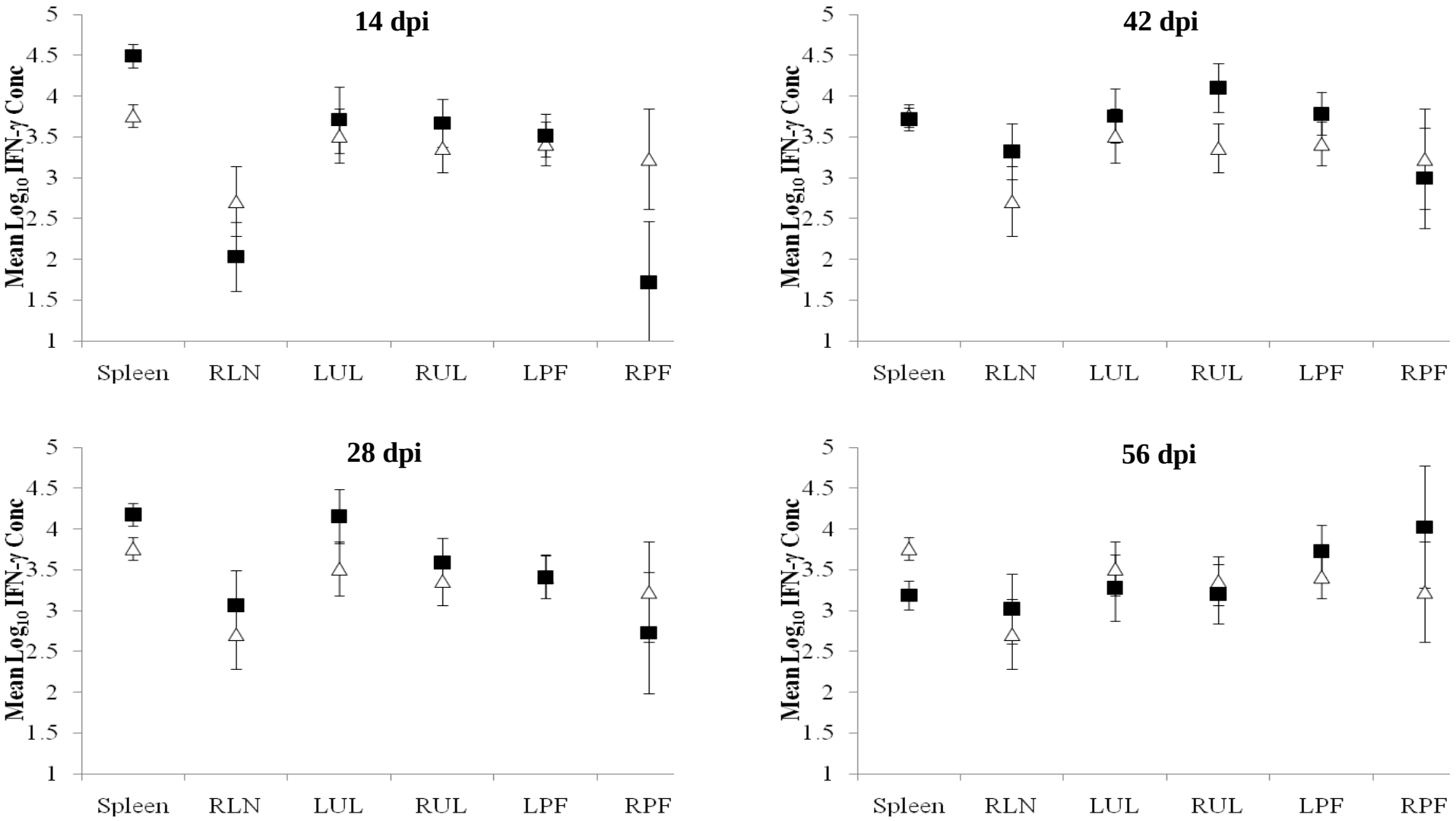


Mean Log_{10} proliferation data from maternal lymph node and spleen samples following stimulation with NCA for 5 days

Mean Log_{10} proliferation data from maternal lymph node and spleen samples following stimulation with NCA for 5 days. Samples of maternal lymph node and spleen were collected at post mortem examination. Samples were stimulated with NCA for 5 days (37°C in a humidified 5 % CO_2 atmosphere), with 18.5 kBq ^3H Thymidine / well being added for the final 18 h, before being harvested onto glass-fibre filters. The data was Log_{10} transformed before analysis by a linear model.

Infected ■, Control Δ (Error Bars = upper (U) & lower (L) 95 % confidence intervals (CI))

Additional File 2. Mean Log_{10} transformed IFN-γ data from maternal lymph node and spleen samples following stimulation with NCA for 4 days

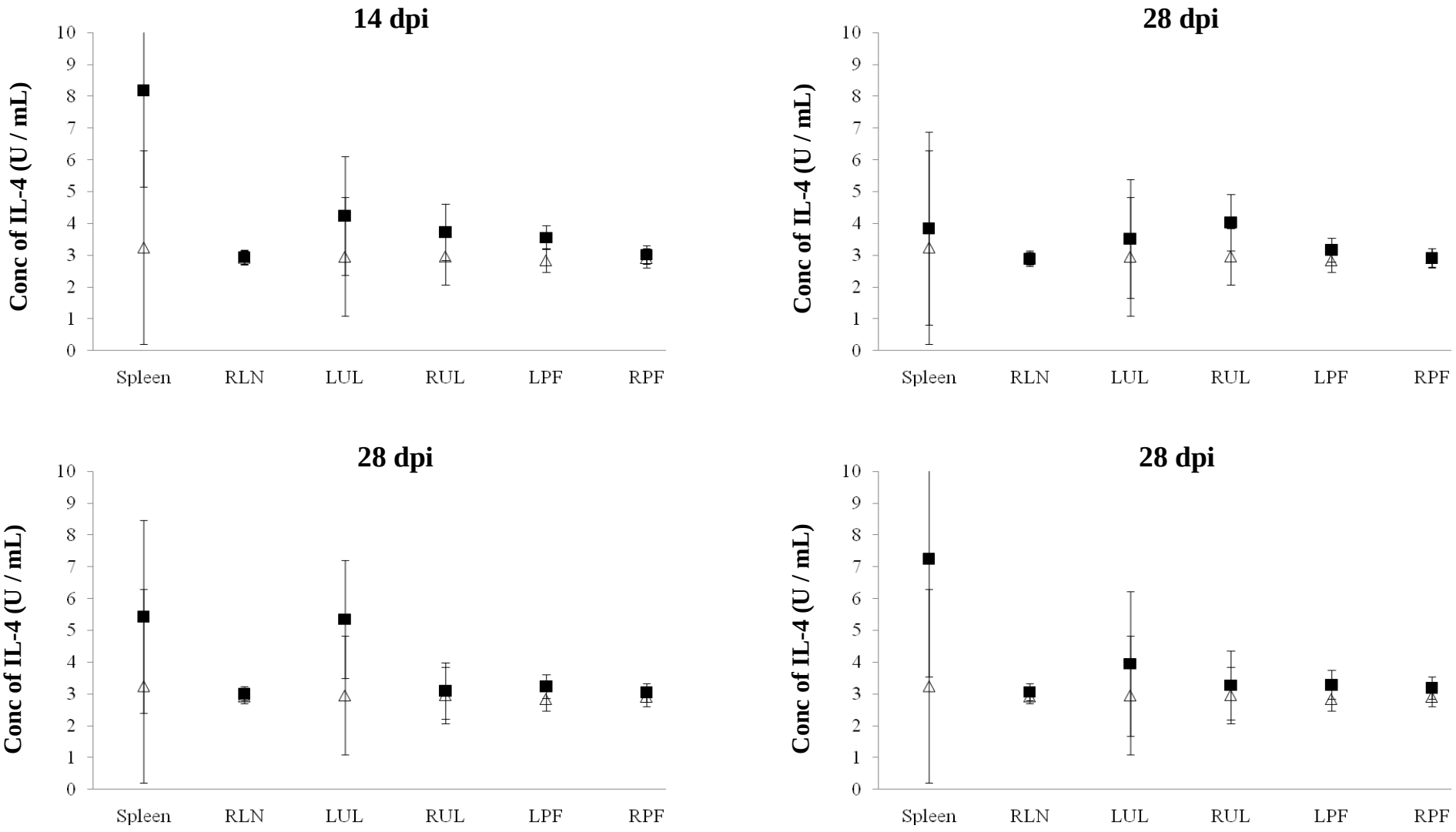


Mean Log_{10} transformed IFN-γ data from maternal lymph node and spleen samples following stimulation with NCA for 4 days.

Maternal lymph node and spleen samples were collected at post mortem examination. Following stimulation with NCA for 4 days (37°C in a humidified 5 % CO_2 atmosphere) cell free supernatants were harvested, ELISA were performed to determine the concentration of IFN-γ produced. The data was Log_{10} transformed before analysis by a linear model.

Infected ■, Control Δ (Error Bars = U & L 95 % CI).

Additional file 3. Concentration of IL-4 in maternal lymph node and spleen samples following stimulation with NCA for 4 days

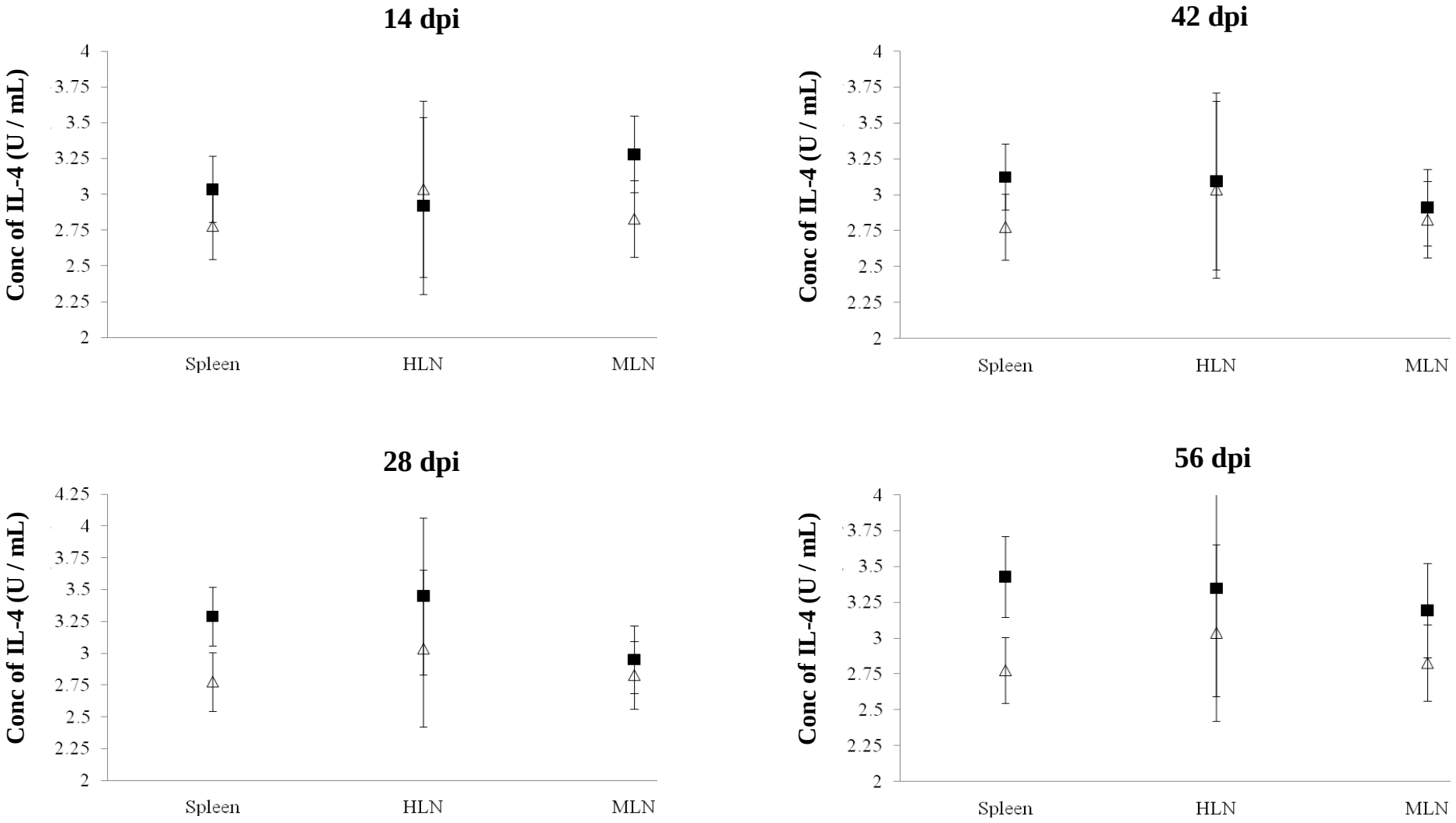


Concentration of IL-4 in maternal lymph node and spleen samples following stimulation with NCA for 4 days

Maternal lymph node and spleen samples were collected at post mortem examination. Following stimulation with NCA for 4 days (37°C in a humidified 5 % CO_2 atmosphere) cell free supernatants were harvested, ELISA were performed to determine the concentration of IL-4 produced.

Infected ■, Control Δ (Error Bars = U & L 95 % CI).

Additional file 4. Concentration of IL-4 in foetal lymph node and spleen samples following stimulation with NCA for 4 days

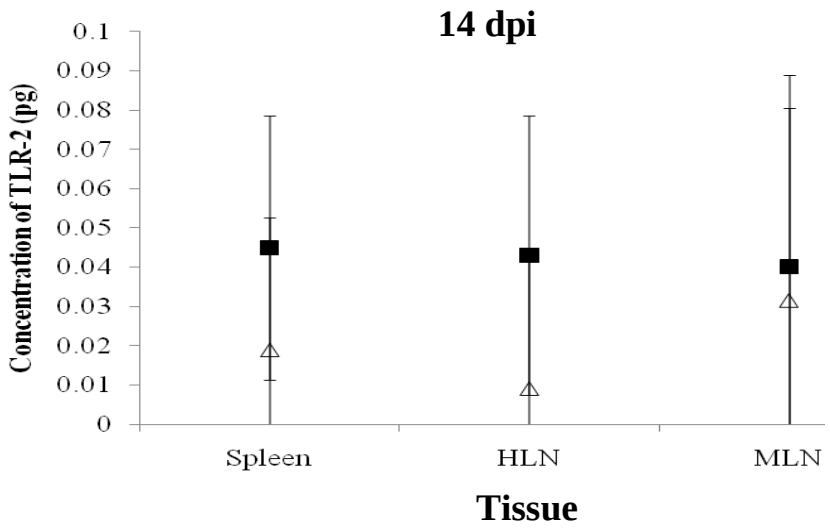


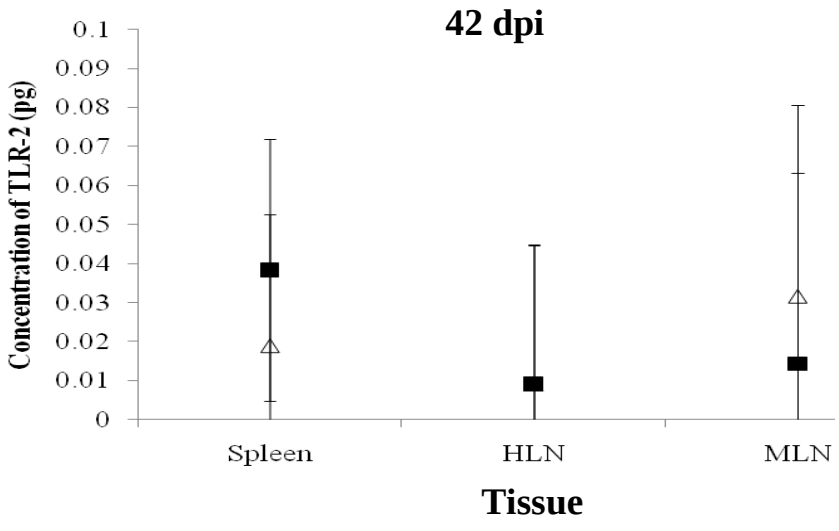
Concentration of IL-4 in foetal lymph node and spleen samples following stimulation with NCA for 4 days.

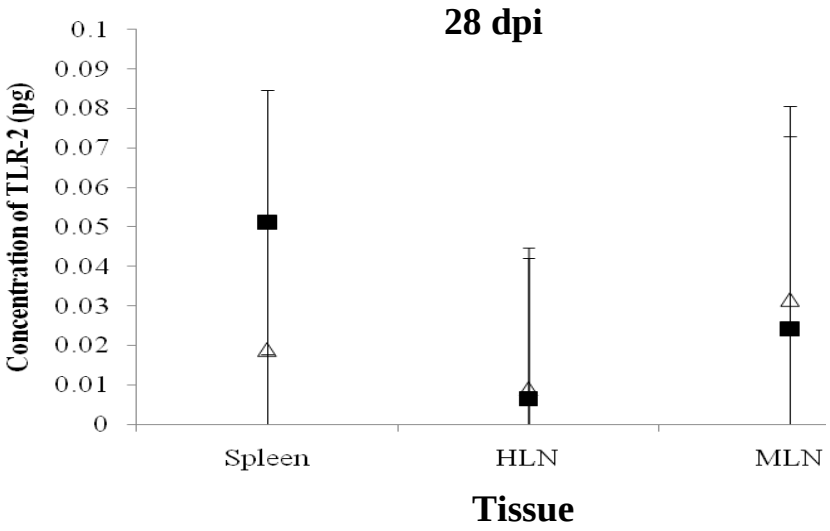
Foetal lymph node and spleen samples were collected at post mortem examination. Following stimulation with NCA for 4 days (37°C in a humidified 5 % CO_2 atmosphere) cell free supernatants were harvested, ELISA were performed to determine the concentration of IL-4 produced.

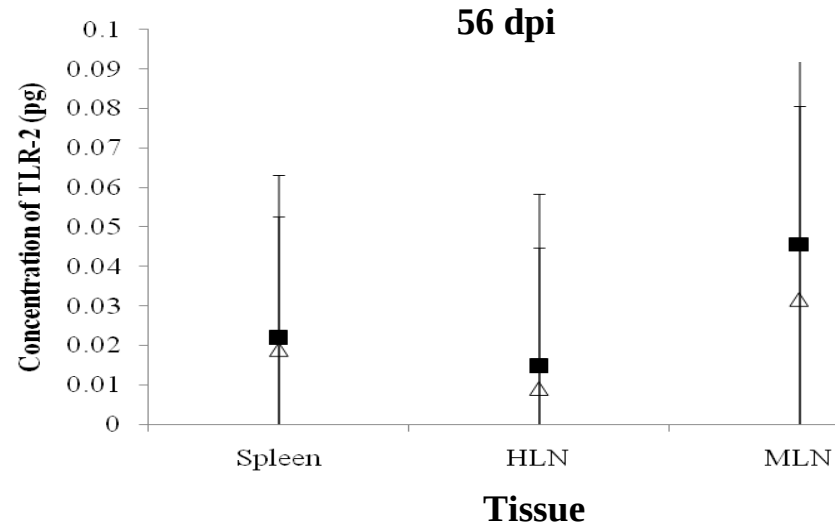
Infected ■, Control Δ (Error Bars = U & L 95 % CI).

Additional file 5. Levels of expression of TLR-2 in foetal spleen, HLN and MLN samples









Levels of expression of TLR-2 in foetal spleen, HLN and MLN samples

Samples of foetal lymph node and spleen were collected at post mortem examination and snap frozen on dry ice. RNA was extracted and used to synthesise cDNA. Levels of expression of TLR-2 were examined with data being normalised against GAPDH expression, results are expressed in pg.

Infected ■, Control Δ (Error Bars = U & L 95 % CI).

8 General discussion

8.1 Major findings

Neospora caninum is considered to be one of the most commonly diagnosed causes of bovine abortion worldwide, costing the farming industry an estimated 1.3 billion US$ per annum in production losses (Reichel et al., 2012). There are currently no chemotherapeutics licensed for use in cattle that will completely resolve a *Neospora* infection, so once a cow becomes infected it remains persistently (chronically) infected for life and can pass the infection to its offspring over several successive pregnancies. These issues make the development of an effective vaccine against *Neospora* highly desirable. However to produce an efficacious vaccine we must first better understand the host–parasite interactions and immune responses involved in protection against infection with the *Neospora* parasites, in order to produce an appropriately targeted control strategy.

The major findings of the studies included in this thesis have helped further our knowledge and understanding in a number of key areas of the host-parasite interaction with *Neospora*. Key findings include that prolonged *in vitro* cultivation of *N. caninum* tachyzoites leads to an attenuation of virulence *in vivo*, when tested in an experimental mouse model system (Bartley et al., 2006). Vaccination of mice with these tissue culture attenuated *N. caninum* tachyzoites protected susceptible animals against a known lethal challenge with virulent wild type *Neospora* parasites (Bartley et al., 2008) and that this

protection was associated with the induction of strong cell mediated immune (CMI) and interferon-γ (IFN-γ) responses (Bartley et al., 2009). These CMI responses not only protected against death but also lead to reduced cerebral parasite burdens in vaccinated mice compared to unvaccinated challenged controls (Bartley et al., 2009). However, vaccination of mice with the tissue culture attenuated *Neospora* tachyzoites did not totally limit the parasite associated pathology, as lesions and tissue cysts were still identified in the brains of mice receiving the attenuated parasites.

Infection studies included in this thesis have further established the importance of the maternal immune response in determining the clinical outcome of a primary *Neospora* infection in pregnant cattle; showing that the type, location and timing of the maternal immune responses are critical in controlling the dissemination of the parasite throughout the host (Bartley et al., 2013a, Bartley et al., 2004, Bartley et al., 2012). The use of controlled serial kill experiments in pregnant cattle allowed the importance of the timing of a primary challenge and therefore the gestational age of the foetus on disease pathogenesis to also be demonstrated (Bartley et al., 2013a, Bartley et al., 2004, Bartley et al., 2012). These pregnant cattle experiments illustrate the proficiency of vertical transmission of *Neospora* following a primary challenge in **naïve** hosts. As gestation progresses, foetal immunity matures leading to a lower incidence of foetal death, but conversely an increased rate of parasite vertical transmission is observed (Bartley et al., 2013a, Bartley et al., 2012). These data highlight the need for an effective vaccine to prevent the vertical transmission of the parasite and protect the developing foetus against *in utero* infection.

Results from the experimental infections in cattle included in this thesis not only show the importance of proliferative cell mediated immune responses and the production of the Th1 type cytokine IFN-γ in both dams and foetuses in controlling parasite multiplication (Bartley et al., 2013a, Bartley et al., 2012), but also demonstrated that the anti-*Neospora* immunity is predominantly involves $CD4^+$ T-cells. The T-cells respond to immunodominant tachyzoite antigens, which are predominantly either parasite surface antigens or proteins that are involved in cellular adhesion and invasion. These include the peptides SAG1, SRS2, GRA2, GRA7, MIC3, and MIC11. Though MIC11 had been previously been described, the work included in this thesis has shown MIC11 to be immunostimulatory and potentially important in protection against *Neospora* (Rocchi et al., 2011).

This project was also the first work to show the involvement of the toll like receptors (TLRs) in innate immune responses in pregnant cattle following an experimental challenge with *Neospora*. Both the dams and foetuses were shown to be up regulating TLR-2 and TLR-9 expression in PBMC and lymph nodes in response to infection (Bartley et al., 2013a). The findings from the serial analysis of experimentally infected pregnant cattle show that the innate, cell mediated and humoral immune responses are all induced following an infection with *Neospora* (Bartley et al., 2013a). The involvement of all aspects of the immune response in *Neospora* infections in cattle highlights the challenges facing those attempting to develop a vaccine against neosporosis.

Comparing data generated by different research groups can often be problematic, as different parasite isolates, challenge doses and routes of inoculation are used. Also many different aspects of the immune response are measured (e.g. gene expression levels compared to serum immunoglobulins levels, cell supernatants compared to serum samples). Many different technologies (qPCR, ELISA, IFAT, cellular proliferation assays) and criteria are also used to analyse the data. The body of work presented in this thesis provided an ideal opportunity to serially examine large numbers of animals (mice and pregnant cattle) challenged and monitored under similar conditions, then examined using the same criteria, allowing clear comparisons to be able to be made not only between the groups but also across the experiments.

Due to the high economic costs attributed to *Neospora* infections in the cattle industry (over 1.3 billion $US per annum) and the fact that there are currently no chemotherapeutics licensed to treat neosporosis in cattle, making vaccination an alternative control strategy. However due to the complex nature of *Neospora*, the different parasite life cycle stages (tachyzoite, bradyzoite and oocysts) and host species (definitive and intermediate) involved in the spread of the disease, leads to the questions of where and when to target a vaccination strategy for the greatest level of control of Neosporosis:

Some of the questions I aim to discuss here include:

- The vaccination of which species would likely have the greatest impact on helping to control the spread of Neosporosis?
- What clinical outcome of a *Neospora* infection in cattle (abortion or vertical transmission) should be targeted for prevention by vaccination for the greatest benefit?
- When would a vaccine need to be administered to be most effective?
- What type of vaccine (live, killed or subunit) would be most efficacious in controlling bovine neosporosis?
- What would the long and short term benefits of an effective vaccine against bovine neosporosis be to farming and the wider community?
- What else needs to be done to help control the spread of *Neospora*? i.e. Biological control and education (an integrated control strategy).

The results and observations contained within this thesis help to address a number of these questions. We are unlikely to ever eradicate *Neospora*, as was achieved with smallpox in the 1980's (Fenner, 1982), or Rinderpest "cattle plague" which was declared eradicated in 2011 (Cima, 2011), as chronic (persistent) *Neospora* infections are known to occur numerous wildlife species including rodents and deer (Dubey, 2003). However a vaccine combined with other long term control strategies such as managing access of dogs (and other definitive hosts) to aborted materials and placenta, rodent control, testing and culling known infected cattle could significantly reduce the economic impact of neosporosis to the farming community.

8.2 The vaccination of which species would likely have the greatest impact on helping to control the spread of Neosporosis?

Though natural *Neospora* infections occur in many species including dogs, sheep and deer (Dubey, 2003), as well as wild carnivores (e.g. foxes and Badgers) (Bartley et al., 2013b), cattle are considered the most economically important host of *Neospora*, with annual losses of over 1.3 billion $US being attributed to foetopathy / abortion caused by neosporosis across both the beef and dairy industries (Reichel et al., 2012). The losses seen in cattle are not all as a result of new (primary) infection, as transmission of *Neospora* from the dam to the foetus can occur as a result of two different infection scenarios. The first is through a primary infection during pregnancy, which leads to the exogenous transplacental transmission (ExTT) of the parasite. This occurs when the dam

becomes infected postnatally for the first time during pregnancy, through exposure to parasites (usually oocysts) in the environment often in contaminated feed and water (Trees and Williams, 2005, Williams et al., 2009). Our experiments demonstrate that this primary exposure of susceptible cattle to *Neospora* during pregnancy can lead to either foetal death (Bartley et al., 2012), or to very high rates of vertical transmission (Bartley et al., 2013a, Bartley et al., 2004), depending on the stage of gestation when the primary infection occurs. The second route of infection is by endogenous transplacental transmission (EnTT), which occurs as a result of the recrudescence (reactivation) of *Neospora* parasites in persistently (chronically) infected dams (Williams et al., 2009). It is this latter condition (EnTT) that is responsible for the majority of foetal infections (Dubey et al., 2007). Davidson and colleagues (1999) calculated that there is a 95% probability that a seropositive dam will produce seropositive offspring (Davison et al., 1999a) (i.e. that vertical transmission will occur), combined with the fact that the parasite can vertically transmit over several successive generations (Bjorkman et al., 1996) shows the scale of the problem facing those attempting to develop effective control strategies for bovine neosporosis.

The work presented in this thesis clearly demonstrates the ease with which the parasite can be vertically transmitted from dam to foetus. Though our experiments show that exposure to *Neospora* during pregnancy can have fatal consequences for the foetus, they also show that cattle are capable of mounting protective immune responses that can stop the vertical transmission of the parasite (Bartley et al., 2012). The presence of a protective maternal response indicates that a vaccine is a viable option for controlling

neosporosis in cattle. The feasibility of a vaccine producing protection against a primary challenge with *Neospora* was confirmed through early experimental studies in cattle carried out at the Moredun Research Institute, where it was demonstrated that exposure of animals to *Neospora* prior to pregnancy lead to protection against vertical transmission, following a parasite challenge during pregnancy (Innes et al., 2001).

The vaccination of dogs would help reduce the environmental contamination with oocysts, which are most likely the cause of abortion storms (Cavalcante et al., 2011). The vaccination of young farm dogs, in particular those that have access to raw bovine tissues (aborted foetuses and placentae) would help reduce the spread of *Neospora* by preventing primary infections in the definitive host and therefore preventing the shedding of oocysts into the environment. However, the results from this thesis along with the available data from field studies and other experimental infections would suggest that vaccinating cattle would have the greatest impact on controlling neosporosis and the economic losses to farmers.

8.3 What clinical outcome of a *Neospora* infection in cattle (abortion or vertical transmission) should be targeted with vaccination for the greatest benefit?

To answer this question we need to look at the dynamics of *Neospora* infections in cattle. Though the most visible outcome of a *Neospora* infection in cattle is the abortion of the foetus, the data from natural and experimental cases of bovine neosporosis would suggest that vertical transmission is actually more important, as it allows for the maintenance of *Neospora* within a herd perpetuating the economic losses. It is undeniable that abortion storms (point source outbreaks leading to mass ExTT events) have a profound and significant financial impact when they occur on a farm (Thurmond et al., 1997, Wouda et al., 1999a). However, following an abortion storm there is also an increased risk that the parasite will be endemically retained within a herd (Moen et al., 1998), when EnTT will then likely become the main cause of foetal infections. So if you could stop vertical transmission (either EnTT or ExTT), you are by analogy also stopping the potential for *Neospora* induced abortions. A vaccine that only prevents the clinical symptoms of infection (i.e. abortion) may be easier to achieve, but is still going to leave lifelong persistently (chronically) infected animals that are capable of repeatedly transmitting the parasite to their offspring.

The experimental data in three cattle experiments presented in this thesis (Bartley et al., 2013a, Bartley et al., 2004, Bartley et al., 2012, Macaldowie et al., 2004, Maley et al., 2003a) showed that post natal infections during early pregnancy (Day 70 of gestation)

resulted in foetal deaths in 56.2% (9/16) of infected animals (Bartley et al., 2012), while vertical transmission was seen in 14/14 infected dams at mid gestation (Bartley et al., 2004, Maley et al., 2006) and in 12/12 dams challenged at late gestation (Bartley et al., 2013a, Benavides et al., 2012). This means that across the three pregnant cattle experiments we saw a foetal death rate of 16.7% (7/42), but an overall 88.1% (37/42) rate of vertical transmission in infected animals. Our findings are comparable to those of other researchers, Williams and colleagues (2000) and Gibney and colleagues (2008) both demonstrated high rates of foetal death following a *Neospora* challenge during early gestation and high levels of vertical transmission at late gestation, but no foetal deaths (Gibney et al., 2008, Williams et al., 2000). The high levels of vertical transmission seen in our data indicates the ease with which *Neospora* could be maintained within a herd, with little obvious clinical symptomatology. It is in the parasites best interest to vertically transmit and not cause abortion, as this allows the parasite to survive and propagate itself with relative ease, where following an abortion the parasite would potentially have the opportunity to undergo sexual replication in a definitive host, but for this to occur the parasite needs to be consumed by a susceptible host to continue its life cycle. It appears that *Neospora* has naturally evolved to be less pathogenic in cattle than other closely related parasites like *T. gondii*, which is more likely to cause an abortion in sheep than to be vertically transmitted and cause a persistent infection (Buxton et al., 2007). The work by Reid and colleagues (2012) on the *Neospora* genome and transcriptome indicates that *Neospora* is missing a number of key genes that have been associated with virulence in *T. gondii* (including ROP18), but contains a greater repertoire of surface antigens than *Toxoplasma* (Reid et al., 2012). It

has been suggested that many of these extra surface antigens are associated with the bradyzoite stage of the parasite. As bradyzoites contain antigens that have not been produced during the tachyzoite life cycle stage they will not immediately be recognised by the host immune response allowing the parasite to evade detection and persistently infect a host, where it can remain immunologically quiescent for long periods of time.

The data from the studies included in this thesis combined with that collected from other experimental infections would suggest that in an ideal world a future control strategy for bovine neosporosis would target vertical transmission. However the reality of the situation is that a vaccine that controls for abortion / foetal death in cattle is more likely to be achievable; especially given the very high rates of vertical transmission demonstrated in this thesis (Bartley et al., 2013a, Bartley et al., 2004, Bartley et al., 2012) and the fact that the parasite can vertically transmit across successive generations (Bjorkman et al., 1996). Strong peripheral maternal immune responses produced during early pregnancy have been shown able to stop foetal death (Bartley et al., 2012). So a vaccine that could stimulate a strong CMI response, particularly during the early stages of pregnancy when the foetus is most vulnerable could prevent foetal deaths and abortions.

8.4 When would be the most effective / appropriate time to administer an anti-*Neospora* vaccine?

A factor that must be considered when developing a vaccine to control bovine neosporosis is the regulation of the maternal immune response during pregnancy in persistently infected animals, parasite recrudescence and vertical transmission across the placenta occur during the down regulation of the maternal cell mediated Th1 type responses during mid pregnancy (Innes et al., 2001). This means that a vaccine would likely need to be administered to susceptible dams either prior to pregnancy to give an appropriate and proportionate protective immune response time to develop before the immunomodulation of pregnancy occurs. The protective response would also need to be maintained throughout the entire of pregnancy, because as the data in this thesis has shown, should a primary infection of susceptible animals occur at mid – late gestation there is a very high risk of parasite vertical transmission (Bartley et al., 2013a, Bartley et al., 2004). Any immune response induced by vaccine should to be rapid, as well as a long lived, as data from Bartley and colleagues (2004) demonstrated that vertical transmission of *Neospora* can occur within 14 days of infection (Bartley et al., 2004).

The timing and location of any maternal immune responses are critical in controlling parasite dissemination, as is the type of immune response generated. Data presented in this thesis (Chapter 6) showed that following a *Neospora* challenge at early gestation, dams carrying live foetuses elicited a stronger Th1 type immune response in peripheral and uterine lymph nodes than the dams carrying dead foetuses (Bartley et al., 2012).

However examination of the same groups of animals by Maley and colleagues (2006) and Canton and colleagues (2013, 2014a and 2014b) showed that high levels of immune cell infiltration ($CD68^+$ macrophages, $CD4^+$ and $CD8^+$ T-cells, γδ T-cells and NK cells) and strong pro inflammatory cytokine (IFN-γ, IL-12 and TNF-α) responses occurring around the placenta were detrimental to foetal survival (Canton et al., 2013, Canton et al., 2014a, Canton et al., 2014b, Maley et al., 2006). The data produced by Maley et al (2006) and Canton et al (2013, 2014a and 2014b), where Th1 type immune responses are detrimental to foetal survival appears to be contradictory to the data presented in Chapters 5-7, which shows the importance of Th1 type responses. However, these results combine show that any control strategy needs to produce a strong peripheral immune response which can control the parasite before it reaches the placenta, where a strong cell mediated immune response can cause severe immunopathology and foetal death.

The data in chapter 6 shows that a immune response biased more towards a Th2 type response (in particular interleukin 4 (IL-4) production) can also lead to an increase in disease severity. Such observations were made in cattle, where PBMC from dams carrying dead foetuses (challenged at day 70 gestation) produced higher Th2 type responses (IL-4 and IL-10) and lower Th1 type (IFN-γ), compared to the dams carrying live foetuses (Bartley et al., 2012).

All of these data combined indicate that successful control of *Neospora* will require a vaccine that promotes a finely balanced Th1 / Th2 response, which allows parasite control without the producing immune associated pathology.

8.5 What type of vaccine formulation (live, killed or subunit) would be most efficacious in controlling bovine neosporosis?

When examining the available data not only within the field of neosporosis but across the broader field of apicomplexan parasitology, it would appear that live parasites offer the best opportunity to produce an effective vaccine (Innes et al., 2011, Reichel et al., 2015). Ideally for a parasite to be considered for use as a live vaccine against bovine neosporosis it would need to satisfy a number of criteria including:

1. The vaccine needs to produce a long lasting protective immune response.
2. Prevent parasite dissemination and the formation of tissue cysts by infecting strain.
3. Demonstrate no reversion to virulence, even under immunosuppressive conditions (i.e. pregnancy)
4. Not lead to a persistent (chronic) infection of the vaccine strain following inoculation.
5. Most importantly in the case of *Neospora*, ensure there is no vertical transmission of the vaccine strain parasites from dam to foetus.

The precedent for using live vaccines to control apicomplexan parasites was set over five decades ago when *Eimeria tenella* was attenuated through serial passage in embryonated eggs (Long, 1965), which lead to the development of the vaccines Paracox / Livacox which are licensed for use against *Eimeria* in chickens (Shirley and Bedrnik, 1997). While Toxovax (the incomplete S48 strain of *Toxoplasma*) is licensed for use against Toxoplasmosis in sheep (Buxton, 1993). A recent study at the Moredun Research Institute, has demonstrated that pigs vaccinated with S48 strain *Toxoplasma* tachyzoites prior to a heterologous oocysts challenge showed a reduction in parasite burdens in muscle tissues used for human consumption (Burrells et al., 2015). As undercooked pork is considered a major source of *Toxoplasma* infections in humans, vaccination could reduce parasite burdens in meat and improve food safety (Dubey, 2008). A live attenuated *Babesia* vaccine is also available to prevent "Tick fever" in Australia (Bock et al., 2004). Other live attenuated parasite species which are showing promise as experimental vaccines include *Leishmania* (Daneshvar et al., 2003), and *Plasmodium* (Vaughan and Kappe, 2013), both of which produced high levels of protection in recipient hosts.

Live attenuated vaccines are likely to induce the most appropriate immune response against *Neospora* as they mimic natural infections. This protective immunity against *Neospora* involves humoral, innate, CMI response and Th1 type cytokines, which are predominantly produced by CD4+ T-cells, this has been demonstrated not only in mice but also in pregnant and non-pregnant cattle (Bartley et al., 2004, Bartley et al., 2009, Rocchi et al., 2011). There is currently little data from live *Neospora* vaccine studies in

cattle, to date only three strains of *Neospora* that have shown natural attenuation have been tested as possible vaccine candidates in cattle. These include the NC-6 Argentina strain which reduced vertical transmission by 75% (Hecker et al., 2013) and Nc Spain-1H, which when administration prior to pregnancy has been shown to reduce foetal mortality by 50% following a heterologous challenge at early gestation. However, the Nc Spain-1H was unable to prevent vertical transmission following a challenge at mid gestation (Rojo-Montejo et al., 2013) and has also been shown to vertically transmit when administered to cattle during early gestation (Rojo-Montejo et al., 2009a). In field conditions administering the live NcIs491 *Neospora* isolate tachyzoites to *Neospora* sero-positive pregnant cattle during mid gestation resulted in a 39% reduction in abortion, but no information was available from this trial about the effect of NcIs491 vaccination on levels of vertical transmission (Mazuz et al., 2015).

If we consider the vaccine potential of the attenuated high passage NC1 parasites described in chapters 2-4 in this thesis, they are capable of protecting against a lethal challenge (Bartley et al., 2008, Bartley et al., 2006) and inducing a strong immune response (Bartley et al., 2009). However they are also still capable of causing a persistent (chronic) infection in a dose dependent manner (Bartley et al., 2008) and though it remains to be confirmed, it is highly likely that at their current level of attenuation the high passage NC1 parasites would also vertically transmit.

Though attenuation of *in vivo* virulence by prolonged serial passage in tissue culture has clearly been demonstrated in chapters 2-4 in this thesis (Bartley et al., 2008, Bartley et

al., 2006, Bartley et al., 2009), the actual mechanisms of the attenuation remains unknown. There is currently no information available on the biological mechanisms involved in the *in vitro* attenuation of *Neospora* parasites, but we may be able draw analogy from *Toxoplasma gondii* and other protozoan parasites. Nischik and colleagues (2001) carried out comparative proteome analysis of attenuated and virulent *Toxoplasma* parasites, showing reductions in the expression of actin, catalase, microneme protein 5 and several dense granule (excretory) proteins in the attenuated parasites, compared to the wild type virulent parasites (Nischik et al., 2001). In *Theileria annulata* the down regulation in the expression of matrix metalloproteinase 9 has been attributed to attenuation in parasites that had undergone an extended period of *in vitro* culture (Adamson et al., 2000).

However we must also use caution when making these comparisons between parasite species, as the work by Reid and colleagues (2012) demonstrated *Neospora* and *Toxoplasma* show some profound differences in their transcriptomes. Just because a protein has been shown to have a function in one species does not mean it will have the same function in another, a prime example is ROP18, which has been shown by a number of research groups to be associated with virulence in *Toxoplasma* (Behnke et al., 2015, Lei et al., 2014), but ROP18 is absent in *Neospora* (Reid et al., 2012). For researchers to more fully understand virulence in *Neospora*, direct genomic and proteomic comparisons need to be made of clonal populations of virulent and attenuated *Neospora* parasites to determine differences in protein coding genes and their subsequent expression. An ideal candidate for this would be the virulent and attenuated

parasites described in this thesis (Bartley et al., 2008, Bartley et al., 2006, Bartley et al., 2009), as they both originated from the same source and have been maintained under the similar conditions. The only clear differences in the virulent and attenuated parasites demonstrated to date are differences in the rates of *in vitro* multiplication and their *in vivo* pathogenicity (Bartley et al., 2008, Bartley et al., 2006, Bartley et al., 2009).

Other factors that need to be considered when looking to produce live vaccines would include:

1. The production costs of a live vaccine will likely be much higher than that of a killed or subunit vaccine.
2. Live vaccines will also have a shorter shelf life than killed / subunit vaccines, which could possibly be dried / lyophilised and reconstituted when required.
3. Live vaccines would likely need cold chain storage to keep the parasites viable, again this would not be required for killed parasites.
4. Finally and probably most importantly live parasites can undergo reversion to virulence causing persistent infections (Bartley et al., 2008, Bartley et al., 2006, Bartley et al., 2009).

Killed / subunit vaccines may at first glance appear to be a more appealing option for controlling bovine neosporosis. They would likely be cheaper to produce, easier to store, have longer shelf life and offer no opportunity for reversion to virulence. However,

sadly the experimental and field data from cattle would suggest that killed vaccines just do not work. The only commercially available killed *Neospora* vaccine Neoguard® has recently been withdrawn from the market. Evidence from field trials using the Neoguard® vaccine showed that it actually increased the risk of vertical transmission and could also have lead to an increase in embryonic deaths (Weston et al., 2012), though no reasons for this increase in susceptibility are stated, it is suggested by Weston and colleagues that the vaccine may be adversely effecting the balance of the host-parasite relationship. Many killed / subunit vaccines have been tested in experimental mouse models with varying degrees of success (Baszler et al., 2000, Cannas et al., 2003a, Debache et al., 2009, Jimenez-Ruiz et al., 2012, Liddell et al., 2003), however we must be careful when using mouse immunological data for drawing conclusions for cattle, as the biology, physiology and immunology of the two species are very different. Very few killed / subunit vaccines have ever made it to testing in cattle. and those that have, like the NC-6 Argentina strain native antigen extract (Hecker et al., 2013) and the polyvalent cocktail containing the recombinant proteins rNcSAG1, rNcHSP20 and rNcGRA7 which both failed to prevent foetal infection (Hecker et al., 2014). The polyvalent cocktail also appeared to promote a severe inflammatory cellular infiltration and cytokine response in the placentomes of vaccinated heifers (Hecker et al., 2015). The work in this thesis examining native tachyzoite antigens clearly demonstrated a number of immunodominant peptides including SAG1 and GRA7 (Rocchi et al., 2011). However it must be remembered that immunodominant does not necessarily mean immunoprotective. Other killed/subunit vaccines have merely been shown to cause no ill effect, like the tachyzoite lysate antigen using a soy-based aqueous adjuvant

(sNcAg/AVEC) (Mansilla et al., 2015) or promote an IFN-γ response in PBMC from non pregnant cattle, like the NcGRA7 entrapped in liposomes coated with mannotriose (M3-NcGRA7) (Nishimura et al., 2013). The fact that no killed / subunit vaccines have succeeded in protecting against vertical transmission in cattle would suggest that the killed vaccine are not stimulating a protective CMI response, this is likely due to the manner in which the killed vaccine antigens are processed by APC's and presented to the immune system, compared to live vaccines which appear more likely to stimulate a protective CMI response similar to those seen during natural infections.

So even though live vaccines have disadvantages, these I feel are outweighed by the overriding fact that they actually work. killed / subunit vaccines may have good efficacy data, but fail at the most important point, none have been shown to stop the vertical transmission of *Neospora* parasites in cattle and some can actually make the situation worse.

8.6 What would the short and long term benefits of a vaccine against neosporosis be to farmers and the wider community?

The most obvious short term gain seen directly by the farmers would be an increase in the productivity of their animals, by reducing abortions, foetal deaths and early neonatal mortality. This in turn would likely also result in a reduction in calving intervals, as *Neospora* negative animals are more likely to complete a pregnancy than *Neospora*

infected animals (Waldner et al., 2001b). In an experiment described in this thesis, four of the pregnant animals challenged iv on day 70 were empty at post mortem on days 42 and 56 post challenge (Bartley et al., 2012). These animals were confirmed pregnant at day 36 of gestation by ultrasound (Macaldowie et al., 2004), so we know foetal loss occurred after this time. However on a farm, foetal loss (reabsorption) could occur due to neosporosis before the pregnancy is confirmed, where a farmer may assume the animal had either not taken at service (not been pregnant) or was infertile. Poor reproductive performance has been shown to be a major reason for culling on dairy farms in the UK (Esslemont and Kossaibati, 1997). Animals that were *Neospora* free would also have a greater saleable value, as *Neospora* infections in cattle have been shown to reduce the value of breeding stock (Trees et al., 1999). A *Neospora* vaccine may also improve the general health of the calves, if animals are not born persistently infected (potentially weakened) they will be healthier, which may allow them to more easily control other common infections. This improved general health may in turn lead to reduced farm costs, as healthier animals will gain weight more rapidly allowing either quicker finishing times and / or better body condition for beef herds at slaughter.

Some of the long term implications of a *Neospora* vaccine would be an overall reduction in the numbers of aborted foetuses, which will lead to a reduction in the levels environmental contamination of parasite. This in turn will reduce the chances of susceptible dogs (and other definitive hosts) from acquiring the parasite through consuming infected foetal tissues (Cavalcante et al., 2011) and shedding oocysts which would lead to a reduction in abortion storms.

In Britain there is probably less of a concern regarding the involvement of wild canids in the sylvatic cycle (non domestic animal cycle) of *Neospora*, as our only known definitive host is the dog, though badgers, foxes and other wild carnivores are known to be infected with *Neospora* (Bartley et al., 2013b) there is no evidence for their involvement in the spread of disease. The sylvatic cycle may be more important in countries where canids such as wolves, coyotes and dingoes are present as well as large populations of stray / feral dogs, as all of these species have been shown to act as definitive hosts for *Neospora* (Dubey et al., 2011, Gondim et al., 2004c, King et al., 2010, McAllister et al., 1998). However when considering the sylvatic cycle we must also consider the involvement of wild / feral rodents, as house mice (*Mus musculus*), field mice (*Apodemus sylvaticus*) and brown rats (*Rattus norvegicus*) have all been shown to be naturally infected with *Neospora* (Ferroglio et al., 2007, Jenkins et al., 2007). The natural hunting behaviour of dogs is to catch and eat rodents, so if you could reduce infections in farm rodents you would by proxy reduce the sylvatic infections.

8.7 What factors may deter farmers from using a *Neospora* vaccine?

The most obvious consideration would be the efficacy of the vaccine, does it work well? If a vaccine has poor performance, then a farmer may consider that the risk of abortion and vertical transmission outweighs the time and expense of administering the vaccine. A vaccine that was able to protect against vertical transmission of the parasite would

need to be used on young naïve animals, to stop the transmission of the parasite to the next generation of animals. So there would be a lack of immediate effect which may also deter users, data would be needed to demonstrate the long term benefits of using such a vaccine on improving reproductive rates. However, a vaccine to prevent abortion could also be effectively used on naïve and persistently infected dams and would show more immediate effects by reducing reproductive losses.

For a vaccine to work it must be implemented as part of a wider integrated farm management strategy. We must educate both farmers and the general public (particularly dog walkers) to help them to understand not only what the parasite is and the disease it causes, but also how the parasite is transmitted and practical precautions that can be taken to try to limit the spread of the disease (i.e. cleaning up after their dogs even on pastures and not letting dogs eat rodents and aborted foetal material and placentas). Farmers can also try and limit disease spread by not using known *Neospora* infected cattle as breeding stock or even culling out known infected animals, if practicable. However there is also need for improved diagnostics for bovine neosporosis. Current commercially available serological tests rely on tissue culture derived tachyzoites or tachyzoite antigens. However antibodies against these tachyzoite antigens can drop to undetectable levels in naturally infected animals, leading to false negative results. This was the case for a control animal in the day 210 gestation experiment (Bartley et al., 2013a, Benavides et al., 2012). Antibody levels against *Neospora* tachyzoites in cows can fluctuate depending on the physiological state of the animal (i.e. pregnant vs. non-pregnant) (Guy et al., 2001) and the activity of the parasite itself (Innes et al., 2007),

while antibodies to the bradyzoite stage of the parasite have also been shown to be highly variable between individual animals (Aguado-Martinez et al., 2008). There is also an alternative explanation for why some animals appear seronegative, though actually infected with the parasite and that is that the animals are immunotolerant to the parasite. Immunotolerance may be due to a foetus becoming infected before its immune system is fully formed, so it is not immunocompetent and does not recognise the parasite as foreign and therefore it does not produce an immune response against it. Though this occurrence is not likely to be common, it could account for a proportion of seronegative cattle that produce pre-colostrally seropositive offspring and would make these animals very difficult to identify.

9 Future work

Work that I would like to carry out in the future to help further progress our understanding of the host – parasite interactions of *Neospora* include:

The studies in mice described in this thesis, showed that after 70 passages in tissue culture, attenuation of virulence was present in *Neospora* tachyzoites. Further prolonged passage of the parasite *in vitro* may reveal increased or even complete attenuation of virulence (causes no pathology), or may even result in creation of an incomplete strain, which is unable to persist (form tissue cysts) within a host when tested *in vivo*. If different isolates were used for vaccination and challenge, then microsatellite DNA

could be used to differentiate between the isolates and determine whether any persistent infections identified are being caused by the vaccine or challenge strain.

Wide gaps exist in our knowledge concerning the initiation of the innate immune responses in cattle following a challenge with *Neospora* and how those early responses drive the induction of a protective CMI response. It has been shown in this thesis that strong peripheral immune responses in cattle are able to inhibit the vertical transmission of the parasite following a challenge during early gestation (Bartley et al., 2012). However this experiment gave no data on the initiation of the immune response, as PBMC were only collected 7 days post challenge and lymph node and spleen samples were collected from day 14 post challenge. The priming of an immune response is likely to play a critical role in whether vertical transmission of the parasite occurs. Comparing the differences in immune initiation in pregnant cattle (at all three stages of gestation (early, mid and late) may allow us to determine why vertical transmission was inhibited at early gestation, but not at mid and late gestation. Phenotypic characterisation of the responding cells would also help direct vaccine development, by illustrating the interactions that occur between cell populations. Longitudinal studies of infected pregnant and non pregnant cattle could also be very informative in answering a key question. What changes immunologically during pregnancy in cattle that allows persistent *Neospora* infections to reactivate?

In order to identify genes associated with virulence and attenuation, metagenomic and proteomic analyses of naturally virulent isolates (those known to be pathogenic in cattle)

and attenuated isolates (those known to show reduced pathogenicity in cattle) of *Neospora* from around the world could be conducted. This should include isolates that have shown attenuation and virulence in experimental models (i.e. *in vitro* attenuated and temperature sensitive mutants). Deep genomic and proteomic analysis could reveal which genes and proteins are up and down regulated. Immunological screening of these peptides using lymphocyte proliferation assays (Rocchi et al., 2011) and IFN-γ production assays (ELISA, qPCR) would indicate which are immunoreactive. The genome editing tool CRISPR-CAS9 could be used to silence genes of interest and demonstrate their involvement in an active infection, virulence and pathology. This information could be used to make a more informed choice for target antigens for use in subunit vaccines.

Metagenomic and proteomic analysis could also be used to screen bradyzoites and tachyzoites, to identify more stage specific genes and proteins, as this information could be used to create a subunit vaccine containing unique recombinant antigens from each life cycle stage. Targeting multiple life cycle stages may improve the vaccine efficacy, as it would allow the host to mount immune responses against the parasite not only during the acute (tachyzoite) stage of infection, but may also allow the host to target the bradyzoites. If the host could reduce the numbers of parasites recrudescing, it may be able to inhibit vertical transmission; this however will only occur if the antigens are presented correctly to the host immune system. In addition, recombinant versions of bradyzoite specific proteins could potentially be developed into a sensitive and specific diagnostic test (i.e. ELISA). Using combinations of antigens i.e. BSR4 and SAG4 in

tandem with tachyzoite antigens may further increase the sensitivity of the assay, as antibodies to all antigens are not always present in the serum. An effective, sensitive ELISA could be used for large scale screening of beef and dairy herds, as well as in the sheep and goat populations. Though neosporosis is mainly considered an infection of cattle, natural infections are also found in smaller ruminants. This information would enable us to get a more accurate estimation of the true sero-prevalence of persistent *Neospora* infections in Britain's domestic ruminant populations.

10 Conclusions

Prolonged *in vitro* cultivation (tissue culture) of *Neospora caninum* tachyzoites can lead to an attenuation of *in vivo* virulence, when tested in an experimental mouse model.

Prior exposure (vaccination) with tissue culture attenuated *Neospora* tachyzoites not only protects against a known lethal challenge with the parasite in mice, but also reduces the morbidiy, mortality and parasite DNA loads in the tissues of vaccinated compared to infected control animals.

Vaccination with tissue culture attenuated *Neospora* tachyzoites leads to the production of a protective immune response. This protection involves both humoral and cell mediated responses, with lymphoproliferative and IFN-γ responses being critical, while IL-10 production may help limit the immunopathology caused by the Th1 type response.

Suggesting, that a balance is needed in the responses that control a *Neospora* infection and those that help limit immunopathology.

The route of inoculation has a profound impact on the clinical outcome of a *Neospora* infection in cattle. An iv compared to a sc challenge was shown to produce more severe disease in bovine foetuses during early gestation.

By day 84 of gestation, bovine foetal lymphocytes are capable not only of mitogenic proliferation, but also produce IFN-γ, IL-4, IL-10 and IL-12 following mitogenic stimulation. On day 98 of gestation one foetus from an iv infected dam which was found dead *in utero* mounted a humoral (IgG) response against *Neospora*, (IFAT titre 1:128). However should the vertical transmission of the parasite occur during early gestation, the foetus is too immunologically immature to mount a protective immune response and foetal death will likely occur.

The stage of gestation when a primary challenge with *Neospora* occurs is important to disease outcome. By mid and late gestation the bovine foetus is capable of mounting protective innate, humoral and cell mediated responses against *Neospora,* helping limit the clinical severity of disease, compared to infections earlier in pregnancy. However, infections during late pregnancy may lead to persistently infected, but otherwise normal healthy calves.

A combined approach of cellular screening and proteomic characterisation of *Neospora* water soluble tachyzoite antigen has allowed the streamlining of the screening of biologically reactive antigen fractions, thus reducing the number of potentially interesting molecules, to a manageable size. Each protein that is characterised can be further investigated through lymphocyte proliferation assays and IFN-γ production for its involvement during a *Neospora* infection

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